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Modulação da resposta imunológica na infecção por micobactérias: implicações na protecção e patologia

Tese de Mestrado
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Abstract

Effective control of *Mycobacterium tuberculosis* infection requires the induction of inflammatory T helper (Th) 1 responses and avoidance of tissue damage. However, little is known about the mechanisms that rule the fine balance between protection and pathology in this infection. The anti-inflammatory cytokine IL-10 is important for the regulation of the immune response to several pathogens. However, despite the fact that elevated levels of IL-10 have been detected in tuberculosis patients, the role of IL-10 during *M. tuberculosis* infection is not clearly understood.

In this study, we evaluated the impact of a transient over-expression of IL-10, during either the innate or the adaptive phases of the immune response, in the outcome of infection. For this, we used a novel animal model, the PMT-10 mice, which over-express IL-10 under the control of a zinc-inducible promoter. These transgenic animals were infected with *M. tuberculosis*, either via the intranasal or intravenous route and the expression of IL-10 was induced at different stages of the infection.

Over-expression of IL-10 early after infection did not influence the capacity of PMT-10 mice to control bacterial growth, regardless the route of inoculation. In contrast, over-expression of IL-10 during the chronic phase of infection caused significant effects in the progression of *M. tuberculosis* infection. Interestingly, whereas in intravenous infection, late IL-10 induction resulted in a transient increase in susceptibility of PMT-10 mice to *M. tuberculosis*, in intranasal infection, induction of IL-10 led to an increased resistance of PMT-10 mice that was maintained until the end of the experiment. However, no major differences were observed between induced and non-induced PMT-10 mice concerning the dynamic of cells or cytokine expression during infection that could explain the different outcomes observed.

Although the cellular and molecular mechanisms underlying the differences observed remain unknown, altogether our data suggest that the impact of IL-10 expression during an infection with *M. tuberculosis* is variable. Indeed, we show for the first time that the timing of IL-10 expression as well as the route of inoculation can determine the outcome of *M. tuberculosis* infection. Further understanding of our observations might be useful in the context of immunomodulatory strategies based on IL-10 expression or suppression.

Resumo

O controlo efectivo da infecção por *Mycobacterium tuberculosis* requer o desenvolvimento de respostas inflamatórias do tipo T de ajuda (Th) 1. No entanto, pouco ainda se sabe acerca dos mecanismos que regulam o equilíbrio entre patologia e protecção no contexto desta infecção. A IL-10 é uma citoquina com propriedades anti-inflamatórias e, portanto, importante na regulação da resposta imunológica a patogénios. Contudo, e apesar da IL-10 se encontrar em elevadas quantidades em pacientes com tuberculose, o papel da IL-10 em infecções por *M. tuberculosis* não está completamente esclarecido.

Neste estudo avaliámos o impacto da sobre-expressão transiente de IL-10, nas fases inata ou adquirida da resposta imunológica, na evolução da infecção. Para isso, utilizamos um novo modelo animal, os ratinhos PMT-10, que sobre-expressam IL-10 sobre o controlo de um promotor induzido pelo zinco. Estes ratinhos transgénicos foram infectados com *M. tuberculosis* pela via intranasal ou intravenosa e a expressão de IL-10 foi induzida em diferentes fases da infecção.

A sobre-expressão de IL-10 cedo após infecção não influenciou a capacidade dos ratinhos PMT-10 em controlar o crescimento bacteriano, independentemente da via de inoculação. Pelo contrário, a sobre-expressão de IL-10 durante a fase crónica da infecção por *M. tuberculosis* provocou efeitos significativos na progressão da infecção. Curiosamente, após a indução de IL-10, enquanto na infecção intravenosa, os PMT-10 apresentaram um aumento de susceptibilidade transiente, na infecção intranasal, os PMT-10 exibiram uma protecção superior aos ratinhos não induzidos. Contudo, não se observaram diferenças relevantes entre PMT-10 induzidos e não induzidos, em relação à dinâmica de células e citoquinas analisadas e mais frequentemente expressas durante a infecção por *M. tuberculosis.*

Apesar dos mecanismos celulares e moleculares responsáveis pelas diferenças obtidas permanecerem por explicar, os nossos resultados sugerem que o impacto da expressão de IL-10 numa infecção por *M. tuberculosis* é variável. Assim, demonstramos neste estudo que o momento da expressão de IL-10, bem como a via de inoculação pode determinar o resultado da infecção por *M. tuberculosis*. A compreensão mais dissecada destes resultados poderá constituir uma mais-valia no desenho de novas estratégias de imuno-modulação baseadas na expressão ou supressão da IL-10.

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ABBREVIATIONS

BCG	Bacille Calmette-Guérin
CFU	Colony forming units
CR	Complement receptor
DCs	Dendritic cells
HIV	Human immunodeficiency virus
IFN	Interferon
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
LAM	Lipoarabinomannan
LN	Lymph node
MHC	Major histocompatibility complex
MT	Metallothioneic
MyD88	Myeloid differentiation protein 88
NK	Natural killer
NO	Nitric oxide
PCR	Polymerase chain reaction
PI3P	Phosphatidylinositol 3-phosphate
RNI	Reactive nitrogen intermediates
TACO	Tryptophan-aspartate containing coat protein
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WHO	World health organization

1. INTRODUCTION

1.1. Current status of Tuberculosis – Overview of the disease worldwide

Despite decades of research on chemotherapy for the disease and development of preventive vaccines, tuberculosis remains a leading global health threat.

Tuberculosis kills more people per year than any other single infectious disease. Its causative agent, *Mycobacterium tuberculosis*, is a slow growing bacillus transmitted primarily by the respiratory route, and although it can cause disease in most organs, pulmonary tuberculosis is the most common (1).

Primary infection leads to active disease in only a minority (about 10%) of infected individuals (2) probably due to the lack of initiation of an appropriate immune response. In the remaining 90 % of cases the immune system controls the infection, but the pathogen is not eradicated. This clinical latency, although not contagious, can persist throughout the person's lifetime. However, reactivation of the latent infection can occur due to perturbations in the host immune response, such as co-infection with the human immunodeficiency virus (HIV), malnutrition, or immunossupressive medication (3).

According to the World Health Organization (WHO) 2009 report (4), the incidence of tuberculosis is increasing worldwide and reached 9,27 million new cases in 2007, even though the number of incident cases per capita is modestly declining. The more affected countries, regarding total number of tuberculosis cases, are India (2.0 million), China (1.3 million), Indonesia (0,53 million), Nigeria (0.46 million) and South Africa (0.46 million), explaining why the Asiatic and African continents have the higher fraction of global cases, with 55% and 31%, respectively. Small proportions of cases were reported in Eastern Mediterranean Region (6%), Europe (5%) and America (3%).

Regarding the European continent, most countries experience a steady decrease in the tuberculosis incidence rate over the last few decades, reaching an overall rate of 17 cases per 100,000 population, although substantial increases were observed in Malta, Iceland, Ireland and Greece. In Portugal, the incidence of tuberculosis has been decreasing 7,3% per year since

2003, with its lowest rate of 29,5 cases (per 100 000 population) in 2007. Nevertheless, Portugal is currently the fifth country in Europe with higher case rates of tuberculosis, following Estonia, Bulgaria, Lithuania and Romania (5).

One of the factors closely associated with the increasing incidence of tuberculosis is the HIV epidemic. In fact, 1,37 million of tuberculosis incident cases (14%) are HIV positive, with the African Region accounting for the higher fraction of global cases (79%). More dramatic is the mortality data, revealing that deaths from tuberculosis among HIV patients account for 27% of the estimated 1,7 million tuberculosis deaths that occurred in 2007 (4).

In addition, the emergence of extensively drug-resistant tuberculosis seriously increases the concerns with regard to transmission and propagation of the disease. Among the 9,27 million episodes of tuberculosis, an estimated 0,5 million cases of multidrug resistant tuberculosis were reported, being India, China, Russian and South Africa the countries with largest number of cases (4). Moreover, in Europe, drug-resistant tuberculosis constitutes the strongest determinant of death among tuberculosis patients (6).

The currently vaccine used against tuberculosis is bacilli Calmette-Guerin (BCG), a live attenuated strain of *M. bovis*. This vaccine, developed around the turn of the last century and first administered in 1921, is given to a large proportion of newborn infants throughout the world, however, the protection that BCG confers against tuberculosis is both incomplete and variable (7).

Thus, it is expected that an increased understanding of disease pathogenesis will contribute to the design of more effective vaccines and new therapeutic strategies (like immunotherapy), which will certainly improve the outcome of individual patients and limit the spread of *M. tuberculosis* around the world.

1.2. Immune response to *M. tuberculosis* – Effector mechanisms

1.2.1. Innate immune response

The differences, among individuals, in the outcome of infection with *M. tuberculosis* may, in part, be explained by the variable efficiency of various innate host defense mechanisms. Immune recognition, phagocytosis, cytokine production and effector mechanisms all contribute to innate immunity.

Alveolar macrophages, residing in the distal airways, avidly engulf inhaled bacteria and are the first cells to be infected by *M. tuberculosis* (8). A variety of phagocytic receptors are involved in the binding and internalization of the bacteria, demonstrating the complex structure of its cell wall structure.

M. tuberculosis can invade host macrophages after opsonization with complement factor C3, which is followed by binding and uptake through complement receptor 1 (CR1), CR3, and CR4 (9). Blocking CR drastically reduces binding and invasion of *M. tuberculosis* but does not abolish it, suggesting that other receptors participate in their uptake. Consistent with this, *M. tuberculosis* has also been demonstrated to bind to the mannose receptor, which recognizes terminal mannose residues on mycobacteria (10). This occurs more frequently with virulent strains of *M. tuberculosis*, suggesting that this route of entrance is advantageous to the bacillus. In fact, phagocytosis through the mannose receptor does not induce anion superoxide production by macrophages (11), and instead triggers anti-inflammatory signals (12). *M. tuberculosis* can also be internalized through the type A scavenger receptor and Fcγ receptors that result in respiratory burst, and in an inflammatory type of response by macrophages (13).

The receptors involved in the phagocytic entry may lead to differences in signal transduction, immune activation and, thus, have a major impact on the survival chances of *M. tuberculosis* once inside the macrophage. However, most of these interactions have been demonstrated *in vitro*, and their relative importance *in vivo* remains to be revealed.

Once internalized, *M. tuberculosis* resides in the phagosome that fuses with early endosomes, arresting iron and other nutrients important for mycobacteria survival (14). Thereafter, *M. tuberculosis* products either located in the cell wall, like lipoarabinomannan (LAM), or released to the cytosol, as the phosphatase SapM and the kinase PKnG, are able to impair the transfer of the phagosome to the lysosome (15-17), where bacterial degradation occurs. The inhibition of phagosome-lysosome fusion by *M. tuberculosis* is due its capacity to interfere with host glycolipids and proteins that regulate this process, such as PI3P (phosphatidylinositol 3-phosphate) (18), TACO (Tryptophan-aspartate containing coat protein) (19) and LRG-47 (47-kilodalton guanosine triphosphatase) (20). As a result, *M. tuberculosis* is able to survive within a phagosome only mildly acidified (21).

The vesicle trafficking events also deliver *M. tuberculosis* to endosomes-related compartments for antigen degradation and subsequent binding to major histocompatibility complex (MHC) class II molecules. Peptide-loaded MHC-II molecules are shuttled to plasma membrane and presented to T cells, initiating a specific immune response (22). However, in vitro studies show that *M. tuberculosis* is also able to subvert this pathway. Indeed, prolonged infection of macrophages with *M. tuberculosis* inhibits the expression of MHC-II and class II transactivator (CIITA), (23, 24) by blocking chromatin remodeling at the CIITA promoter (25), thus resulting in decreased MHC-II antigen presentation.

Besides expressing phagocytic receptors, macrophages and dendritic cells (DCs) also express several pattern recognition receptors, such as Toll-like receptors (TLRs) that recognize specific molecular patterns expressed by pathogens. All the TLRs have, at least, one signaling pathway dependent on intracellular adaptor molecule myeloid differentiation factor 88 (MyD88) (26). Thus, MyD88 deficient mice ($MyD88^{-/-}$) are highly susceptible to aerogenic *M. tuberculosis* infection, implying that TLR signaling plays an important role in mycobacteria resistance (27). A major consequence of *M. tuberculosis* interaction with TLRs is the secretion of cytokines and chemokines that activate the macrophage and regulate the development of an antigen-specific adaptive immune response.

The 19 kilodalton (kDa) lipoprotein, a secreted antigen of *M. tuberculosis*, was the first ligand shown to interact specifically with TLR2 to induce tumor necrosis factor (TNF) and nitric oxide (NO) production from both murine and human macrophages (28). Other in vitro studies show that the immunostimulatory responses to *M. tuberculosis* LAM, and phosphatidylinositol

mannoside (PIM) are also mediated by TLR2 (29, 30). But interestingly, despite a large collection of TLR2 agonists present on the tuberculosis bacillus, *in vivo* studies in the mouse model indicate that TLR2 is not essential for host resistance against low dose *M. tuberculosis* infection (31-33). Besides TLR2, other TLRs may be involved in immune recognition of *M. tuberculosis*. An undefined heat-labile cell-associated mycobacterial factor was found to be a ligand to TLR4 (30), however, $TLR4^{-/-}$ mice do not show a compromised resistance to tuberculosis following an aerosol challenge (32, 34). Also, TLR9 was shown to bind GpG dinucleotides in bacterial DNA, inducing a rapid antimycobacterial response in macrophages, in a phospholipase D-dependent manner (35). Therefore, the understanding of the in vivo role played by of the various TLRs in the host defense against *M. tuberculosis* infection awaits further experimentation.

As it was mentioned above, recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and induction of cytokine production, which itself induces further macrophage activation and cytokine production, in a complex dynamic process in order to kill the mycobacterial infection.

One of the most important pro-inflammatory cytokines produced by macrophages in response to *M. tuberculosis* infection is TNF. The role of this cytokine is of great clinical relevance as TNF-blocking drugs, used as anti-inflammatory therapy to rheumatoid arthritis, are associated with reactivation of latent tuberculosis in humans (36, 37). Also, in mouse models, disruption of the TNF gene or neutralization of TNF lead to disorganized granulomas, a characteristic and crucial feature of tuberculosis, compromising the containment of *M. tuberculosis* (38). Importantly, TNF, along with the cytokine interferon gamma (IFNγ) produced mainly by T-lymphocytes induce NO production in macrophages by activating the inducible nitric oxide synthase (iNOS) enzyme (39). NO reacts with oxygen radicals forming reactive nitrogen intermediates (RNI) that can kill or inhibit *M. tuberculosis* growth. However, in some cases, and despite the high toxicity of these products, *M. tuberculosis* is able to persist within this environment. This resistance is based on multiple strategies developed by the bacilli, such as the mycobacterial proteasome and the production of KatG, a catalase-peroxidase that can inactivate RNI within phagosomes (40, 41).

Finally, TNF can also induce apoptotic cell death in macrophages during *M. tuberculosis* infection (42). By denying infected bacilli a protected intracellular environment for bacillary

replication, TNF-mediated apoptosis represents an innate defense mechanism that slows the increase of bacterial load following infection. In fact, several studies associate the virulence of *M. tuberculosis* strains with apoptosis (43-45), indicating that virulent strains actively suppress apoptosis by interfering with TNF signaling and by up-regulating the expression of anti-apoptotic molecules, such as Mcl-1 (46).

It is therefore accepted that *M. tuberculosis* persistence in the host is, in part, due to its cell wall resistance and to the capacity of bacilli to modify effector functions of macrophages and DCs. However, some inhibitory mechanisms can be intentionally initiated by the host to counteract detrimental side-effects of a pro-inflammatory response. Whether triggered by the host or by *M. tuberculosis*, the macrophages and DCs are able to produce anti-inflammatory cytokines, such as IL-10. The specific role of IL-10 during *M. tuberculosis* infection will be discussed in section 1.3. However, tumor growth factor (TGF-β) is also an anti-inflammatory cytokine that, like IL-10, is produced during the chronic phase of *M. tuberculosis* infection (47, 48), suggesting an important immunomodulatory role for this cytokine.

Beside macrophages and DCs, neutrophils and natural killer (NK) cells also participate in the innate immune response.

NK cells are recruited early during *M. tuberculosis* infection and are a primary source of IFNγ, either in response to IL-12 or IL-18 production by macrophages and DCs, reinforcing the bactericidal capacity of these cells (49). Unlike T-cells, NK cells do not recognize mycobacterial antigens presented by MHC class I or class II molecules. Whether these cells are essential to innate resistance to *M. tuberculosis* is not completely proved (50, 51).

Circulating neutrophils are also recruited to the lungs early after infection. Their role in host defense against *M. tuberculosis* is supported by studies showing that depletion of neutrophils early after intravenously challenge with *M. tuberculosis* compromises the immune response against mycobacterial infection (52-55). Moreover, an *in vitro* study suggested that the phagocytosis of apoptotic neutrophils by macrophages decreases the viability of intracellular *M. tuberculosis,* due to the uptake of neutrophil antimicrobial peptides (56). Yet, the mechanisms by which neutrophils can mediate antimicrobial activity against *M. tuberculosis* are not completely understood.

In the majority of individuals exposed to *M. tuberculosis*, the innate response alone cannot protect from infection, and effector T cells of the adaptive immune response are necessary to restrict bacterial growth and to mediate protection. Therefore, following *M. tuberculosis* phagocytosis and concomitant TLR activation, the next step in the development of host immunity is the transport of pathogen from the lung to the draining lymph node (LN), where the process of adaptive immune response is initiated.

1.2.2. Adaptive immune response

After aerosol exposure, DCs infected by *M. tuberculosis* are capable of migrating to the LN where naive T cells can be activated (8). This activation requires expression of antigen in the context of MHC, costimulatory molecules, and the necessary cytokines that promote T cell differentiation (57).

The antigen specific T helper (Th) 1 cells are induced mainly in the presence of IL-12 (58, 59) and are actively involved in the control of tuberculosis. This CD4 T cell subset is able to produce IFNγ to full activate the antimicrobial mechanisms of macrophages. Murine studies have shown that IFNγ $^{-/-}$ mice rapidly succumb to *M. tuberculosis* infection (60, 61) and antibody depletion of CD4 T cells, similarly, decreases resistance to *M. tuberculosis* infection (62). In humans, the loss of CD4 T cells in HIV patients greatly increases susceptibility to both acute and reactivation tuberculosis (63). In addition, individuals carrying defective genes for IFNγ receptor or IL-12 receptor (64) are more susceptible to intracellular pathogens, including low virulent mycobacteria such as BCG.

Various studies investigated the role of CD8 T cells, during a *M. tuberculosis* infection. These cells are limited to either cytotoxicity activity or secretion of IFNγ (65), which happens when DCs take up apoptotic vesicles containing *M. tuberculosis* antigens and cross-present them on MHC Class I (66, 67). However, the impact of CD8 T cells in the protection against *M. tuberculosis* is not completely understood, as the absence of CD8 T cells in a low dose aerosol model of infection had little impact in disease progression, until after 200 days post infection (68, 69).

Investigators have recently identified Th17 cells as another subset of CD4 T cells that is detected in mouse models and in humans exposed to *M. tuberculosis* (70). The differentiation of this T cell lineage is induced in the murine model by the action of TGF-β and IL-6 (71), but also

requires IL-23 (72) to become a persistent population. The first studies on the role of Th17 cells during mycobacterial infection indicate that these cells mediate neutrophil recruitment during inflammatory responses (73, 74), and can be negatively regulated by IFNγ (75). However, a protective role for Th17 in tuberculosis has not been yet demonstrated. It has been shown that Th17 cells are induced after vaccination and promote an accelerated IFNγ response during subsequent *M. tuberculosis* infection (76). Studies in our laboratory, on the other hand, showed that *M. tuberculosis* infected mice, repeatedly challenged with mycobacterial antigen, have an enhanced pulmonary pathology with an increased lesion size that is dependent on both IL-23 and IL-17 (*A. Cruz et al, unpublished*).

The migration of macrophages and of differentiated T cells to the site of infection culminates in the formation of a granuloma. This structure encompasses the bacilli, residing within the macrophages, and functions as an immune microenvironment to facilitate interactions between T cells, macrophages and the produced cytokines (77). In addition, the granuloma serves to wall off the bacteria from the rest of the lung, limiting its spread (78). However, in some cases, *M. tuberculosis* may induce chronic immunopathology leading to lesions that undergo caseation necrosis, that provide excellent conditions for bacterial extracellular growth, and give rise to cavities that allow the bacillus to spread through the airways to other parts of the lung and outside the environment (3).

1.3. Modulation of the immune response: Role of IL-10

1.3.1. IL-10 in infectious diseases and in therapy strategies

During an infectious disease, the host immune system needs to respond with sufficient intensity and duration to control and eliminate the infection. However, strong antimicrobial effector mechanisms can often cause significant collateral damage to the host, which sometimes is more harmful than the infection itself. Thus, the presence of anti-inflammatory cytokines is important for the safe resolution of infection, as it is confirmed by the critical role of IL-10 in a variety of infectious diseases (79).

Thus, it has been shown that reduction of IL-10, using monoclonal antibodies or IL-10$^{-/-}$ mice, during *Plasmodium spp*. (80-82) and *Toxoplasma gondi* infections (83, 84), resulted in the onset of severe or even fatal immunopathology, caused by excessive IFNγ mediated responses. In addition, injection of mice with recombinant IL-10 or over-expression of this cytokine in transgenic mice enhanced their survival during toxic-shock-like syndromes, by reducing the levels of TNF, IFNγ and MIP-2 (85, 86).

However, the presence of anti-inflammatory cytokines can also delay or impair protective immune responses. This is supported by studies showing that reduction of IL-10 decreased the survival of intracellular pathogens, such as *Listeria monocytogenes* (87), *Candida albicans* (88), and *Leishmania major* (89). In addition, excessive or untimely IL-10 production inhibit the pro-inflammatory responses to *T. cruzi* (90), *Plasmodium yoelli* (91), *Leishmania spp* (92, 93) and lymphocytic choriomeningitis virus (94) to the extent that these pathogens escape immune control, which can lead to either fulminate or chronic non-healing disease.

Therefore, the resolution of infection requires a coordinated response in which pro-inflammatory mechanisms clear the pathogen and are then down-modulated by IL-10, before immunopathology occurs. IL-10 has itself to be strictly controlled to avoid an inefficient response or the development of chronicity.

The profound immunosuppressive effects of IL-10 have prompted a variety of clinical studies to employ recombinant IL-10 to treat patients with immune mediated inflammatory diseases. The clinical effects of recombinant IL-10 have, however, been quite heterogeneous. For instance, whereas almost no effect was seen in rheumatoid arthritis, significant response was

observed in psoriasis (95). Patients with psoriasis that received recombinant IL-10 had significant clinical benefits, decreasing the size of psoriatic areas as well as its severity index (96). However, in some other studies with larger numbers of patients and more severe forms of psoriasis, the systemic administration of IL-10 resulted in only temporary clinical improvement but not remission (97). More encouraging results were observed when IL-10 was delivered locally to the area of inflammation rather than systemically (98). In experimental autoimmune encephalomyelitis, an animal model for human multiple sclerosis, *Cua et al* showed that, for optimum therapeutic activity, IL-10 had to be delivered directly to the central nervous system by an adenovirus vector, whereas systemic administration was ineffective (99). This study suggested that the localization and timing of IL-10 production or administration may determine its effectiveness. The local delivery of IL-10 to sites of inflammation has been a major challenge, and new experimental approaches are being undertaken in this area, such as bacteria delivery systems (100) or nanoparticles-in-microsphere oral systems (101).

1.3.2. IL-10 sources and targets

IL-10 is produced by different types of immune cells, from both myeloid and lymphoid lineages (79). Monocytes, macrophages and DCs are important producers of IL-10, generally when induced by TLR2 but also TLR4 ligands (102). B cells are also a potentially important source of IL-10 (103), as are some granulocytes, including eosinophils and mast cells (104). In addition, many subsets of T cells can a produce IL-10, such as Th2 cells, inducible regulatory T cells, which are generated in the periphery, and natural regulatory T cells, which are generated in the thymus (105). Interestingly, Th1 and, more recently, Th17 cells were also found to coproduce IL-10, (106-108) together with IFNγ or IL-17, when induced by particularly strong antigen dose and inflammatory responses, allowing the immune responses to be inherently self-regulating (109).

Most hematopoietic cells express the IL-10 receptor and, therefore, IL-10 is able to down-regulate many steps in the pathway of both the innate and adaptive immunity.

IL-10 produced early in the immune response can act in antigen presenting cells in an autocrine way, down-regulating the expression of MHC class II and the costimulatory molecule B7-1/B7-2 (110, 111). In addition, IL-10 can inhibit the production of pro-inflammatory cytokines, including IL-1α and β, IL-6, IL-12, IL-18, and TNF, as well as the expression of

chemokines implicated in the recruitment of monocytes, DCs, neutrophils, and T cells (MCP1, MCP5, RANTES, IL-8, IP-10, and MIP-2) (79). As a result, the effects of IL-10 in macrophages and DCs indirectly compromise the activation of effector T cells and subsequent initiation of adaptive immunity.

In addition, IL-10 can act directly on T cells to limit their proliferation and production of cytokines, such as IL-2, IFN-γ, IL-4 and IL-5 (112, 113) or may promote the differentiation of naïve T cells into IL-10-producing regulatory T cells (105) that, in turn, may directly influence macrophages, DCs and effector T cells (114).

In contrast, IL-10 also have stimulatory effects on mast cells (115), and in B cells by up-regulating the expression of MHC class II molecules and enhancing IgA responses (114), and induces the recruitment, proliferation and cytotoxic activity of NK cells and CD8 T cells (116).

1.3.3. IL-10 in mycobacterial infections

There are evidences demonstrating that Il-10 is produced in patients with tuberculosis. Indeed, T cells expressing both IFNγ and IL-10 have been isolated from the bronchoalveolar lavage fluid of tuberculosis patients (117), and expression of IL-10 mRNA has been demonstrated in circulating mononuclear cells of the pleural fluid (118). Nevertheless, the role of this cytokine during *M. tuberculosis* infection is not clearly understood. That IL-10 may be important in tuberculosis is suggested by reports associating the risk of developing tuberculosis with the presence of human polymorphisms. For instance, SLC11 A1, a tuberculosis susceptibility locus, has been associated with increased innate IL-10 response that may lead to a tendency towards the development of primary progressive tuberculosis (119). In addition, studies in Turkish and Cambodian populations found a decrease in the frequency of the allele *IL10* – 1082/, related to progression in lung tuberculosis (120, 121). However, in other populations (122-124) no differences in the IL-10 genotype frequencies were observed among tuberculosis patients.

In vitro and *ex vivo* studies indicate that IL-10 inhibit or modulate the immune response against *M. tuberculosis*. Several studies demonstrate that IL-10 antagonizes the activation of macrophages infected with *M. tuberculosis* by down-regulating the production of TNF and IL-12 (125-127), as well as the expression of costimulatory molecules (128). Furthermore, IL-10 inhibits the activation of CD4 T cells and the production of IFNγ in response to *M. tuberculosis* (128-130).

These works suggest that IL-10 may suppress the development of the immune response against *M. tuberculosis*. However, *Jung et al* showed that IL-10$^{-/-}$ mice display an identical capacity to control *M. tuberculosis* infection as wild-type mice, despite the stronger Th1-mediated immunity generated early in the infection (131). In the other hand, it was recently reported that IL-10$^{-/-}$ mice infected with *M. tuberculosis* display exacerbated immunopathology, thereupon succumb at very late stage of infection (132). In contrast, IL-10$^{-/-}$ mice are more resistance to *BCG* infection than wild-type mice (131, 133), probably due to enhanced levels of TNF and iNOS in the granuloma. In addition, IL-10 neutralizing antibodies in *M. avium* infection resulted in enhanced bacterial clearance (134, 135).

Transgenic mice that over-express IL-10 show increased susceptibility to *M. tuberculosis* and other mycobacterial species. However, these works are not consensual in revealing what immune mechanisms are impaired by IL-10, and whether they correlate with the increased susceptibility to mycobacterial infection.

Turner et al. showed that mice over-expressing IL-10 in activated T cells during *M. tuberculosis* infection, exhibited an impaired Th1 development, as characterized by decreased numbers of activated T cells in the blood and lung tissue as compared to wild-type mice (136). However, *Murray et al*, in another T cell-specific IL-10 transgenic model, showed that after BCG infection IL-10 had little effect on T cell function, but rather acted primarily in the costimulatory functions of macrophages (137).

In models in which IL-10 is specifically over-expressed by macrophages, the Th1 response induced upon mycobacterial infection seemed to be unaffected. *Feng et al* (138) and *Lang et al* (139), showed that IL-10 over-expression deactivates macrophage by inhibiting TNF and IL-12 in *M. avium* and *BCG* infection, respectively. However, *Schreiber et al* (140) suggested that after *M. tuberculosis* infection, over-expression of IL-10 does not impair effector cytokines, but instead induces an alternative activation pathway in lung macrophage that inhibits RNI production.

Altogether, the results of these studies are difficult to compare as, besides using different mycobacterial species, other experimental settings diverge, such as the cellular source of IL-10, the route of infection and the time points assessed.

Therefore, further studies are needed to dissect the role of IL-10 during mycobacterial infections. We propose, that before dissecting the precise mechanisms inhibited by IL-10 which impact the outcome of *M. tuberculosis* infection, it is important to firstly identify *when* they occur during the infectious process.

In the previous studies, although the increased bacterial loads detected in IL-10 transgenic mice occurred during the chronic phase of infection, we question whether: (i) it was a consequence of an impairment in the early innate mechanisms that affected long-term cellular responses and, therefore, influenced the disease progression, or (ii) IL-10 was able to hinder the immune system when acquired immunity was established, causing increased bacterial replication. The fact that the transgenic mice models used so far over-express IL-10 constitutively during the infection, hampers any attempt to address these 2 questions. Assessing separately the consequences of IL-10 during these two singular periods of the host immune response against *M. tuberculosis* is, undoubtedly, a more reliable approach that would further allow us to unravel the cause-effect mechanism underlying IL-10 regulation.

For this purpose we used a novel model of IL-10-expressing transgenic mice, the PMT-10 mice, which over-express IL-10 under the control of a zinc-inducible methionine promoter. In this context, we evaluated the impact of a transient over-expression of IL-10, during either the innate or the adaptive phases of the host immune response, in the outcome of *M. tuberculosis infection*.

1.4. Aims

The aim of this thesis is to understand the role of IL-10 in *M. tuberculosis* infection.

We propose to:

- Determine the impact of high expression of IL-10 in the outcome of intranasal or intravenous *M. tuberculosis* infection;
- Distinguish the progression of the disease when IL-10 is over-expressed during the early or late stages of *M. tuberculosis* infection;
- Understanding the mechanisms underlying protection/susceptibility;

2. MATERIAL AND METHODS

Animals. PMT10 animals on a C57BL/6 background were produced by Drs. P Vieira and AG Castro. A mouse IL-10 cDNA sequence was cloned in the p169ZT vector (141), which carries the sheep metallothioneinc (MT) Ia promoter (142), a β-globin splice site and the SV40 polyadenylation signal. The resulting vector (pMT-IL10) was injected in C57BL/6 eggs and transgenic founders were identified by PCR using MT specific primers.

IL-10 over-expression was induced by giving a 2% sucrose solution with 50 mM of zinc sulfate to animals *ad libitum*. As the IL-10 promoter is associated with a metalloprotein, the presence of zinc in this solution induces its activation. Serum levels of IL-10 could be measured 3 days after induction. A group of transgenic littermates were supplied with regular water, as a control. All animals were bred under specific pathogen-free conditions in our animal facilities.

Bacteria and infection. The H37Rv strain of *M. tuberculosis* was grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and prepared for infection of mice with 10^2 colony-forming units (CFU) via the respiratory route or 10^5 CFU via the intravenous route. Before intranasal infection, mice were anesthetized with Ketamine (Imalgene 1000)/Medetomidine (Domitor).

Bacterial load determination. Infected mice were killed by CO_2 asphyxiation and the organs were aseptically excised. Each of the organs was individually homogenized in phosphate-buffered saline (PBS), and serial dilutions of the organ homogenate were plated on nutrient 7H11 medium (Middlebrook). CFUs were counted after 3 weeks of incubation at 37°C.

Cell preparation and culture. Lung cell suspensions were prepared by removing the lung aseptically and sectioning it in ice-cold DMEM (Mediatech-Cellgro) using sterile razor blades. Dissected lung tissue was then incubated in DMEM containing collagenase IX (0.7 mg/ml; Sigma- Aldrich) at 37°C for 30 min. Digested lung, lymph node and spleen tissues were gently dispersed by passage through a 40- μm pore size nylon tissue strainer (Falcon; BD Biosciences);

the resultant single-cell suspension was treated with ACK solution to remove any residual red blood cells, washed twice, and counted. These cells were used for flow cytometric analysis.

Flow cytometry analysis. Single cell suspensions from the lungs or draining LN were prepared as described above and cells were stained with labeled antibodies specific for CD4 (RM4-5), CD11c (N418), I-A/I-E (M5/114.15.2) purchased from Biolegend and CD11b (M1/70), GR-1 (RB6-8C5), CD44 (IM7), CD62L (MEL-14) purchased from BD Biosciences. Cells were analyzed using a BD Biosciences FACSCalibur and data analyzed using FlowJo7 software.

Real-time PCR. Total RNA from cell suspensions was extracted with TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reverse transcription was done with whole RNA in a final volume of 20 µl using SuperScript II (Invitrogen) and Oligo(dT) (Roche) according to the manufacturer's instructions. The cDNA was then subjected to real-time PCR for quantification of ubiquitin, TNF and IFNγ in 10 µl with SYBR Green Supermix (Bio-Rad) in CFX 96 Real-Time (Bio-Rad) system. Primers had the following sequences: ubiquitin sense: 5'- TGG CTA TTA ATT ATT CGG TCT GCA -3', antisense: 5'- GCA AGT GGC TAG AGT GCA GAG TAA -3'; TNF sense: 5'- GCC ACC ACG CTC TTC TGT CT -3', antisense: 5'- TGA GGG TCT GGG CCA TAG AAC -3'; IFNγ sense: 5'- TGGCAAAAGGATGGTGACATG-3', antisense: 5'- GACTCCTTTTCCGCTTCCTGA - 3'. All reactions were performed using the following cycling parameters: 1 cycle of 95°C for 15 min, followed by 3 cycles of 95°C for 15 min, 58°C for 20 min and 72°C for 15 min, and 2 amplification cycles of 65°C for 1.3 min and 95°C for 15min; and 1 cooling cycle of 35°C for 1.3 min.

Cytokine ELISA. The concentration of IL-10 in sera was determined at appropriate dilutions by the mouse IL-10 Ready-SET-Go ELISA kit (eBiosciences)

Histological studies. All excised tissues were fixed in 10% phosphate-buffered formalin. Sections of organs (4 µm) were processed for light microscopic studies after hematoxylin-eosin or Ziehl-Neelsen staining and analyzed using morphometric tool of BX61 Olympus microscope. This tool determines the area defined by the squared pixel value for each granuloma. For

immunohistochemistry, paraffin was removed from the formalin-fixed lung sections, which were then washed with xylene, alcohol and PBS. Antigens were 'unmasked' with Citrate Buffer Heat induced Epitope Retrieval (Lab Vision). Sections were probed with goat anti-mouse specific for inducible nitric oxide synthase (M-19.G; Santa Cruz Biotechnology), which was detected with Alexa Fluor 568–conjugated polyclonal goat anti-rabbit (Invitrogen). DAPI (4¢,6-diamido-2-phenylindole hydrochloride) was used to counterstain tissues and to detect nuclei. Pictures were obtained with a BX61 Olympus microscope.

Statistical analysis. The results are given as means ± SE. Statistical significance was calculated by using Student's t test. Values of $p \leq 0.05$ were considered significant.

3. RESULTS

3.1. Effect of IL-10 over-expression early after infection with *M. tuberculosis*: impact on the innate and acquired immune responses.

Early immunity against *M. tuberculosis* infection encompasses several critical events, as activation of macrophages and DCs in the lung, dissemination of *M. tuberculosis* to the draining LN and local priming of T cells (8). Several studies show that IL-10 is able to down-regulate the activation of macrophages and DCs and, therefore, the generation of Th1-mediated responses (79). In that sense, we questioned whether over-expression of IL-10 during the early phase of the immunological response against *M. tuberculosis* affects the outcome of intranasal or intravenous experimental infections.

In the intranasal infection model, a group of PMT-10 mice, was induced to over-express IL-10 between days 4 and 13 post infection, a period in which bacilli disseminate from the lung to the LN, where priming of T cells starts to occur. As for the intravenous model, i-PMT-10 mice were induced to over-express IL-10 between 3 days before *M. tuberculosis* challenge and 6 days post infection. We decided, in this latter model, to induce IL-10 before infection, as upon intravenous infections, dissemination of bacteria to the spleen and T cell activation occur almost immediately, thus resulting in T cell activation earlier than observed for intranasal infections. The IL-10 promoter was induced by administering, in the drinking water, a solution of zinc sulfate to PMT-10 mice. In both models, two groups were analysed: infected induced PMT-10 (i-PMT-10) and, as a control, infected non-induced PMT-10 littermates.

i-PMT-10 mice transiently over-express IL-10 early after intranasal or intravenous M. tuberculosis infection.

In order to confirm that i-PMT-10 mice, in both models of infection, transiently over-expressed IL-10 early after *M. tuberculosis* exposure, we measured by immunoassay the amounts of IL-10 in the serum of infected animals.

As shown in figure 1, in both models, the levels of circulating IL-10 in control littermates were below the detection limit (BDL) of the assay, at all the time points analyzed. After intranasal

infection (Fig. 1a), IL-10 levels could be detected at day 7 (3 days after zinc-mediated induction) in i-PMT-10 mice, being higher at 13 days post infection (the day of the end of the zinc treatment). At day 16 post infection, as in control PMT-10 mice, circulating IL-10 could no longer be detected in i-PMT-10 mice. Also, in the intravenous infection (Fig. 1b), i-PMT-10 mice showed transient increased IL-10 production, until 6 days after infection.

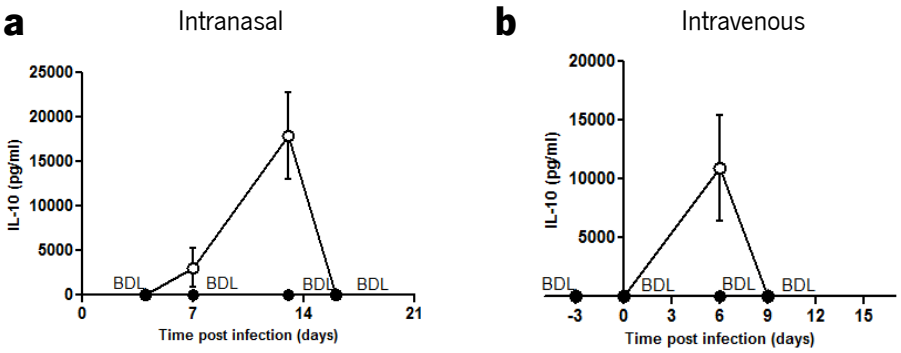

FIGURE 1. i-PMT-10 mice transiently over-produce IL-10 during intranasal or intravenous *M. tuberculosis* infection. i-PMT-10 mice (○) were induced to over-express IL-10 (a) between 4 and 13 days after intranasal *M. tuberculosis* infection, or (b) 3 days before intravenous challenge until 6 days of infection. At indicated time points, IL-10 concentration was determined in the serum of i-PMT-10 and control littermates (●) by ELISA. Data represent the mean ± SEM from four mice per group from one experiment (a) or one representative of two independent experiments (b).

Over-expression of IL-10 during the early immune response against M. tuberculosis does not impact the outcome of either intranasal or intravenous infection.

At various time points after *M. tuberculosis* infection, organs were collected and the bacterial loads were compared between PMT-10 and i-PMT-10. As shown in figure 2a, after intranasal infection, the bacterial load in lungs of non-induced littermates progressively increased till reaching approximately 6 logs on day 28, after which the bacterial growth was controlled at a stationary level, as previously described (143). Lungs from i-PMT-10 mice showed similar level of bacterial burdens than control mice, over the course of infection (Fig. 2a). Similarly, both control

and i-PMT-10 mice infected via intravenous route were equally capable of controlling the bacterial burden in the liver, spleen and lung (Fig. 2b-d).

Our data suggest that an early over-expression of IL-10 does not impair the host resistance to *M. tuberculosis* infection.

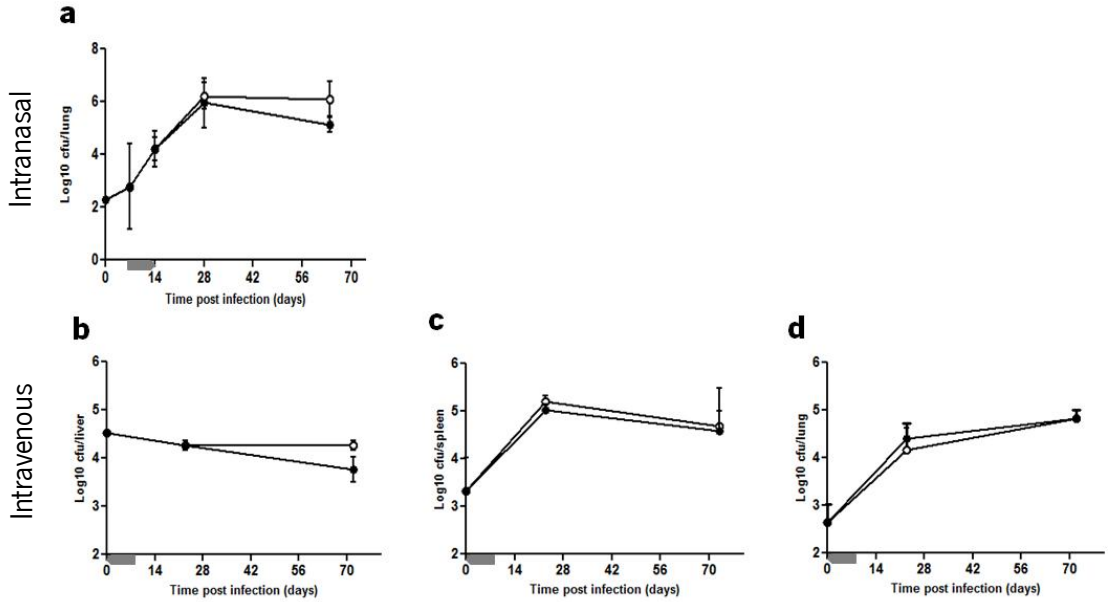

FIGURE 2. Early IL-10 over-expression does not affect the resistance of i-PMT-10 mice to either intranasal or intravenous *M. tuberculosis* infection. i-PMT-10 mice (○) and control littermates (●) were infected intranasally (a) or intravenously (b-d) with 2,3 log10 CFU or 10^5 CFU of *M. tuberculosis*, respectively. i-PMT-10 mice over-expressed IL-10, as shown in figure 1, during the time-frame indicated (grey bar). Lungs (a and d), livers (b) and spleens (c) were removed at the indicated time points, and the number of viable bacteria were determined by plating serial dilutions of organ homogenates on Middlebrook 7H11 medium. Data represent the mean ± SEM from six (a) or five (b-d) mice per group from one experiment (a) or one representative of two independent experiments (b-d).

IL-10 over-expression affects the granuloma size developed after intravenous but not intranasal M. tuberculosis infection.

A protective immune response against pathogens needs to limit tissue damage while clearing the pathogen (144). IL-10 has been clearly associated to the regulation of inflammation and pathology prevention (79). Due to anti-inflammatory properties of IL-10, we were prompted to compare the extension of the inflammatory process in PMT-10 and i-PMT-10 infected with *M. tuberculosis*. For that, infected organs were collected, and morphometric analysis of the lesions was performed.

During the course of intranasal or intravenous *M. tuberculosis* infection, both i-PMT-10 mice and control littermates developed granulomatous lesions. These lesions, typical of mycobacterial infections, are defined as a focal accumulation of mononuclear cells, forming an organized structure with centrally located macrophages surrounded by a lymphocyte cuff (78). Thus, granulomas are thought to afford the T cell-macrophage contact and cooperation necessary for an effective antimycobacterial defense. On the other hand, granulomas displace and destroy adjacent lung tissue and may necrotize at the center leading to cavity formation, which is the most relevant sequelae of the chronic inflammatory response to *M. tuberculosis* (77). Importantly, lungs of i-PMT-10 mice, infected by the intranasal route, presented a granuloma size similar to that of control littermates, as shown in figure 3a. On the other hand, in livers of intravenously infected mice, it was evident that i-PMT-10 mice developed larger granulomas than non-induced PMT-10 mice (Fig. 3b), although histopathological analysis of the livers revealed that the 2 groups of mice developed similar number of granulomas per field after intravenous infection (data not shown).

These data show that an early over-expression of IL-10, while not impacting the bacterial growth in both infection models, induces, in the intravenous infection a larger inflammatory area at later time points.

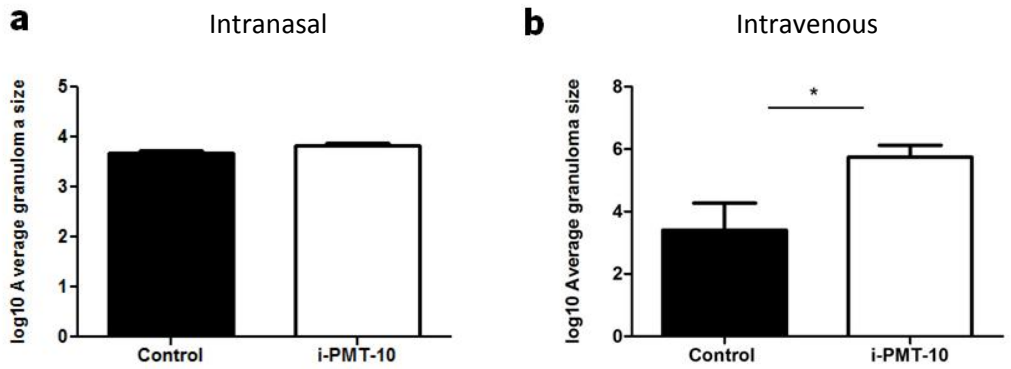

FIGURE 3. Morphometric analysis of the granulomas developed by intranasally or intravenously infected mice. i-PMT-10 mice (white bars) and control littermates (black bars) were infected intranasally (a) or intravenously (b) with 2,3 $\log_{10}$ CFU or 10^5 CFU of *M. tuberculosis*, respectively. At 64 days (a) or 72 days (b) post infection morphometric analysis of granuloma was performed in lungs (a) and livers (b) sections of infected mice. Data represent the mean ± SEM from six (a) or five (b) mice per group from one experiment (a) or one representative of two independent experiments (b). * $p < 0.05$

Early IL-10 over-expression induces a delay in the expansion of several populations of leukocytes in the lymphoid organs of intranasal or intravenously infected mice.

We next investigated whether an intact cellular response was present in i-PMT-10 mice, despite the high levels of IL-10 observed in these animals, during the initiation of the response. To test this, we next evaluated, in the course of intranasal or intravenous infections, the kinetics of the cellular populations, previously described to be inhibited by IL-10.

In the intranasal model, shown in figure 4, the total number of cells in the lungs from both induced and control PMT-10 mice followed similar kinetics over time (Fig. 4a). However, by analyzing, by flow cytometry, separately the major leukocyte subsets (Fig 4b-e), it became evident that the number of CD4 T cells and CD11c cells similar in both groups until day 28 post infection, were significantly increased at day 64 in i-PMT-10 mice (Fig. 4b,d). As for the LN, the total number of cells in control mice increased until day 14 post infection, before undergoing a slow decline until day 64 (Fig. 4f). However, in i-PMT-10 mice, the total number of cells increased at a slower rate and peaked at day 28, sustaining the same amounts throughout the infection.

This delay in i-PMT-10 mice was evident in all cell types analyzed (Fig. 4g-j) and the relative proportion of each cell type was not affected over time (data not shown).

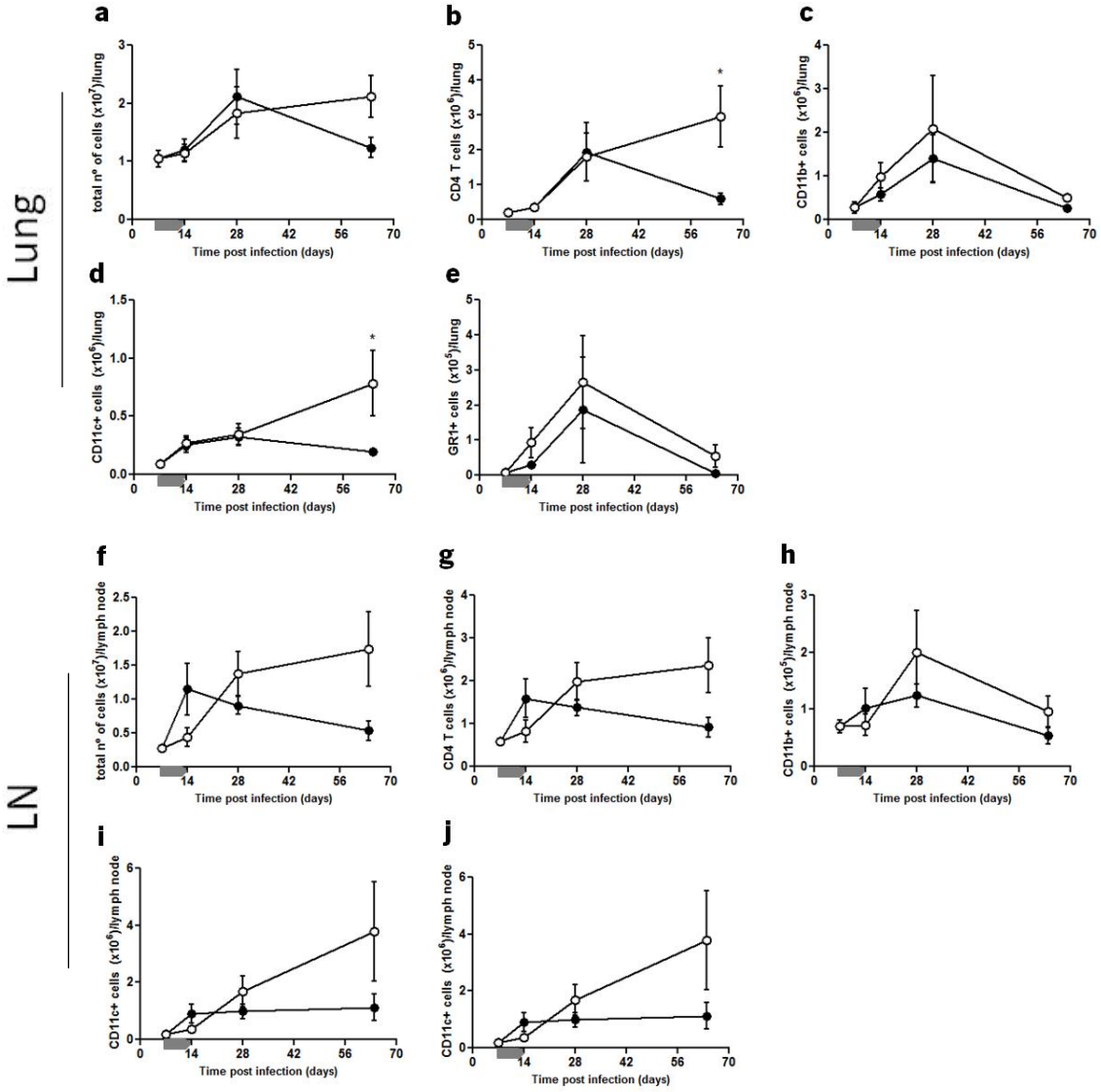

FIGURE 4. Kinetics of cellular expansion in the lungs and LN of intranasally infected mice. i-PMT-10 mice (○) and control littermates (●) were infected intranasally with 2,3 $\log_{10}$ CFU of *M. tuberculosis*. i-PMT-10 mice over-expressed IL-10, as shown in figure 1a, during the time-frame indicated (grey bar). At different time points, single cell suspensions from lungs (a-e) and LN (f-j) were prepared, stained with antibodies specific for CD4, CD11b, CD11c, GR-1 and analyzed by flow cytometry. The total number of cells of lungs (a) and LN (f) was determined using the Newbauer chamber. Data represent the mean ± SEM from six mice per group from one experiment. * $p < 0.05$.

The intravenous model resembled the major features of the intranasal model. As seen in figure 5a, after *M. tuberculosis* intravenous infection, while the total number of cells in the spleen of control mice roughly stabilized after day 21, the number of splenocytes in i-PMT-10 mice was significantly lower at day 21, after which it progressively increased until day 72, reaching the same amounts as in control mice. This difference was observed for the major cell population analyzed, being more pronounced in CD4 T cells and CD11c cells (Fig. 5b,d), in accordance with the observed in the intranasal model of infection. In addition, it seems that after intravenous infection, the increased granuloma size observed in the livers of i-PMT-10 mice at day 72 is not related to an increased cellular response at this time point, suggesting that perhaps IL-10 altered, instead, the organization/conformation of the granuloma.

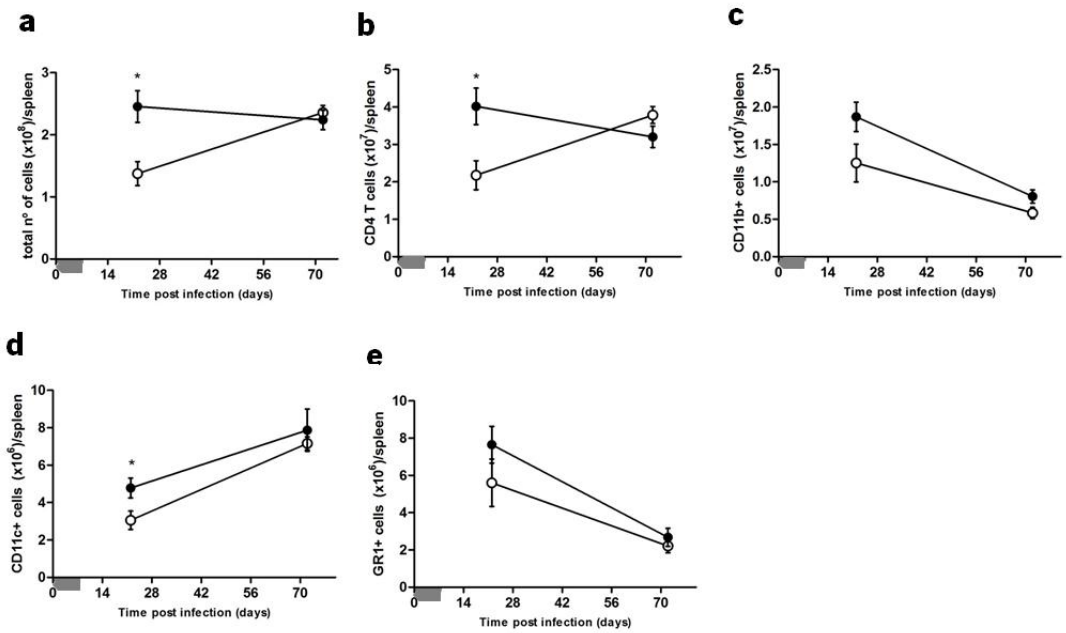

FIGURE 5. Early over-expression of IL-10 induces a delay in the expansion of cells in the spleen of intravenously infected mice. i-PMT-10 mice (○) and control littermates (●) were infected intravenously with 10^5 CFU of *M. tuberculosis*. i-PMT-10 mice expressed IL-10, as shown in figure 1b, during the time-frame indicated (grey bar). At different time points, single cell suspensions from spleens were prepared and stained with antibodies specific for CD4, CD11b, CD11c and GR-1 and analyzed by flow cytometry. The total number of cells of speens (a) was determined using the Newbauer chamber. Data represent the mean ± SEM from five mice per group from one representative of two independent experiments. * $p < 0.05$.

Altogether, our data points out to a potential contribution of IL-10 over-expression to an initial delay on the cellular dynamics observed on the LN of intranasally infected i-PMT-10 mice and on the spleens of intravenously infected i-PMT-10 mice. Of note, this delay is timely related to the peak of IL-10 production observed in i-PMT-10 mice, under our experimental conditions. Interestingly, however, is the later increase of certain cellular populations observed in the intranasal model of infection in the lungs of i-PMT-10 mice. It is tempting to suggest that this increase might be an attempt of the immune response to overcome any down-modulating effects of IL-10.

After intranasal M. tuberculosis infection, early over-expression of IL-10 induces an increased expansion of activated CD4 T cells in both lungs and LN.

It has been described that activation of naïve T lymphocytes in the draining LN, which occurs at about 14 days upon intranasal or aerosol *M. tuberculosis* infection (145), and their subsequent migration to the site of infection, are essential for the induction/potentiating of host defensive mechanisms against *M. tuberculosis* (8). Since the delay observed in intranasally infected i-PMT-10 mice occurred at around day 14 and affected CD4 T cells, we hypothesized that the early over-expression of IL-10, between day 4 to 13 post intranasal infection, could be impairing the activation of CD4 T cells in the LN and lungs of i-PMT-10 mice. To test this hypothesis, we measured, by flow cytometry, the expression of CD44 and CD62L during the course of infection. We found that, in the CD4 gate, the $CD44^{high}$ $CD62L^{low}$ population, indicative of T cell activation, was significantly increased, in both LN and lungs of i-PMT-10 mice at 28 days post infection, as compared to littermates control (Fig. 6). Our results thus suggest that the early expression of IL-10 during *M. tuberculosis* infection, not only impair CD4 T cell activation, when IL-10 expression was at its peak, but instead seemed to potentiate it afterwards. Again, it is possible that this increased emergence of activated CD4 T cells occurred to counteract the delayed cellular expansion detected in the LN of intranasally infected i-PMT-10 mice.

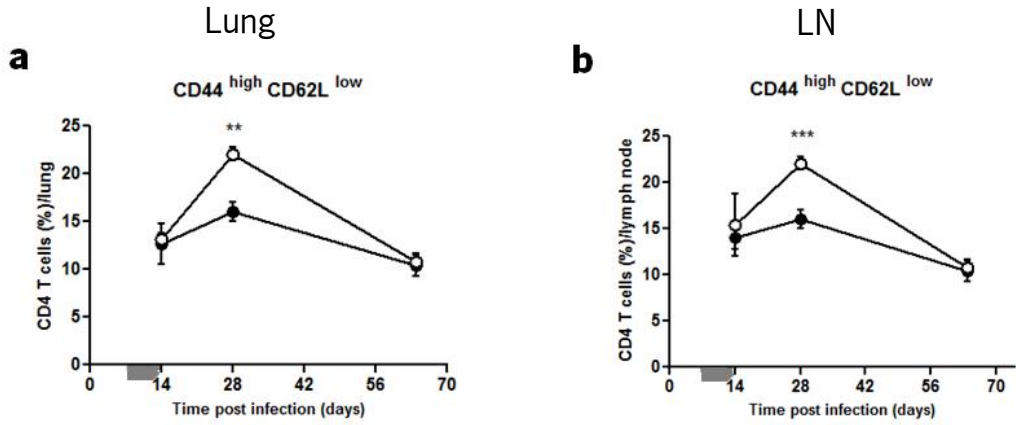

FIGURE 6. Early over-expression of IL-10 induces an increased expansion of activated CD4 T cells at 28 days post intranasal infection. i-PMT-10 mice (○) and control littermates (●) were infected intranasally with 2,3 $\log_{10}$ CFU of *M. tuberculosis*. i-PMT-10 mice were over-expressed IL-10, as shown in figure 1, during the time-frame indicated (grey bar). At different time points, single cell suspensions from lungs (a) and LN (b) were prepared and stained with surface and activation markers for flow cytometry analysis of activated $CD44^{high}$ $CD62L^{low}$ CD4 T cells. Data represent the mean ± SEM from six mice per group from one experiment. ** $p < 0.01$, and ***$p < 0.001$

Early IL-10 does not impair the expression of IFNγ and TNF during the course of infection in either the intranasal or intravenous models.

Several studies demonstrate the requirement of IFNγ and TNF for macrophage activation and initial control of *M. tuberculosis* infection (61, 146). Since i-PMT-10 mice were able to control the infection by *M. tuberculosis*, despite the fact that IL-10 has been extensively described to suppress the production of the former cytokines, we asked whether IFNγ and/or TNF were normally expressed in infected i-PMT-10 mice.

Semi-quantitative real-time PCR analysis showed that, after intranasal *M. tuberculosis* infection, IFNγ expression in the lungs of control mice, started to progressively increase after day 7, reaching a peak at day 28, consistent with the influx of Th1 cells to the lung (8), before undergoing a slow decline until the end of the experiment (Fig. 7a). Infected i-PMT-10 mice showed identical amounts of IFNγ transcripts, in the lungs, over time. As shown in figure 7b, the expression of TNF in control littermates increased after intranasal *M. tuberculosis* infection peaking at day 14, before decreasing to near basal levels. However, in i-PMT-10 mice, TNF expression progressed more slowly until day 14, but continued to increase until day 28, reaching

significantly higher levels than control littermates. Interestingly, this high induction of TNF in the lungs of i-PMT-10 mice occurred at the time of increased activation of CD4 T cells, suggesting that 2 weeks after the IL-10 over-expression period, i-PMT-10 mice reacted with an increased inflammatory response.

After *M. tuberculosis* infection via the intravenous route, expression of IFNγ was highly induced in the spleens from both induced and control PMT-10 mice at day 21, maintaining the same level throughout the infection (Fig. 7c). The kinetics of TNF expression was also comparable in the spleens of both groups of mice (Fig. 7d), showing the inability of IL-10 to impair the induction of pro-inflammatory cytokines after *M. tuberculosis* infection in our experimental settings.

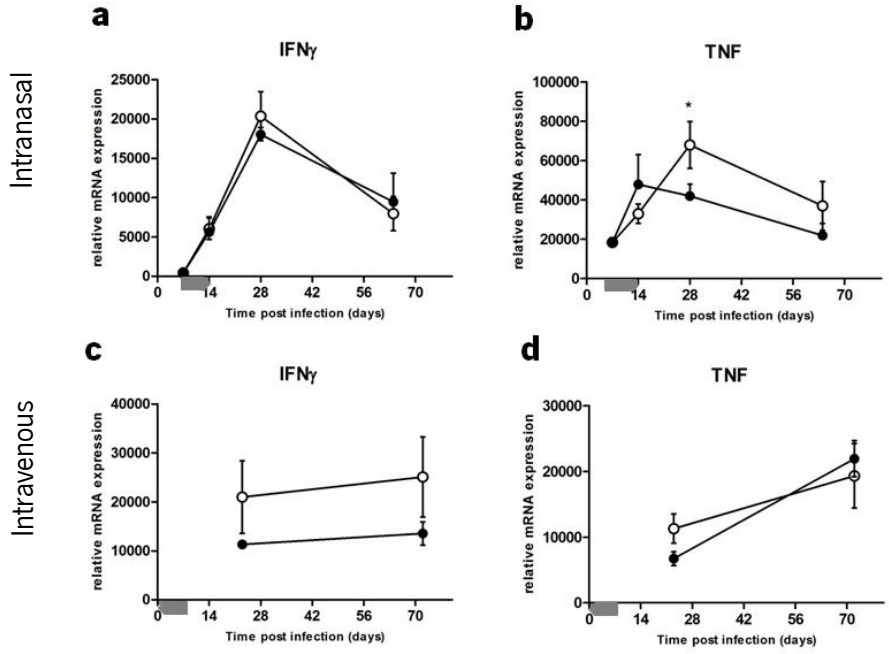

FIGURE 7. Early over-expression of IL-10 does not impair the expression of IFNγ and TNF after intranasal or intravenous infection. i-PMT-10 mice (○) and control littermates (●) were infected intranasally (a,b) or intravenously (c,d) with 2,3 log10 CFU or 10^5 CFU of *M. tuberculosis*, respectively. i-PMT-10 mice over-expressed IL-10, as shown in figure 1, during the time-frame indicated (grey bar). Gene expression of IFNγ (a,c) and TNF (b,d) was determined in lungs (a,b) or spleen (c,d) homogenates by real time PCR. Data represent the mean ± SEM from six (a,b) or five (c,d) mice per group from one experiment (a,b) or one representative of two independent experiments (c,d). * $p < 0.05$.

Taken together, the data presented in part 3.1 suggest that over-expression of IL-10, early after intranasal or intravenous *M. tuberculosis* infection, induces a delayed expansion of lymphoid populations that is not sufficient to affect the outcome of infection, probably due to the fact that IFNγ and TNF were not impaired in the time points analyzed. Regarding the intranasal model, whether the late, but increased, inflammatory response detected in the lungs of i-PMT-10 mice was critical or redundant for the resolution of infection is still not answered.

3.2. Effect of IL-10 over-expression late after infection by M. tuberculosis: impact on the ongoing immune response.

The adaptive immune response generated in the individuals who are exposed to *M. tuberculosis*, although protective, does not induce sterilizing immunity. These individuals, therefore, remain latently infected, and are vulnerable to disease reactivation when their immune surveillance weakens, or when their immune response is compromised (3). Elevated levels of IL-10 were detected in individuals with active tuberculosis (117, 118). However, it is unclear whether IL-10 plays a role in promoting the reactivation of tuberculosis in chronic latent infected individuals. Since IL-10 has been shown to inhibit both T cell proliferation and IFNγ production we decided to investigate whether high amounts of IL-10 in a phase of infection where latency is established could compromise the immune response and increase host susceptibility.

To address this question, PMT-10 mice, infected via the intranasal or intravenous route by *M. tuberculosis,* were induced to over-express IL-10 during the late phase of infection, more precisely between days 53 to 62 in intranasal model, or between days 47 to 56, in intravenous model. At various time points thereafter, IL-10 was measured in the blood by immunoassay (Fig. 8). As expected, in both infection models, whereas IL-10 was not detected in the serum of control animals, in i-PMT-10 mice, circulating IL-10 was detected 3 days after zinc administration, specifically at day 56 or 50 after intranasal (Fig. 8a) or intravenous (Fig. 8b) infection, respectively, and was highly produced until the end of the induction period.

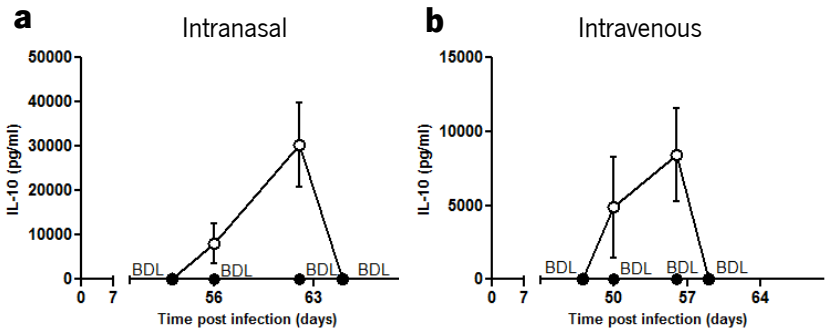

FIGURE 8. i-PMT-10 mice transiently over-produce IL-10 during the late phase of intranasal or intravenous *M. tuberculosis* infection. i-PMT-10 (○) mice were induced to over-express IL-10 between 53 and 62 days (a) or between 47 and 56 days (b) after intranasal or intravenous *M. tuberculosis* infection, respectively. At various time points IL-10 concentration was determined in the serum of i-PMT-10 and control littermates (●) by ELISA. Data represent the mean ± SEM from four mice per group from one experiment (a) or one representative of two independent experiments (b).

IL-10 over-expression during the late phase of M. tuberculosis infection resulted in different outcomes of infection, depending on the route of infection.

At various time points after *M. tuberculosis* infection, organs were collected and the bacterial loads were compared between PMT-10 and i-PMT-10 mice. After intranasal infection, non-induced PMT-10 mice were able to control the bacterial burdens in the lung during the course of infection, as expected (Fig. 9a). Surprisingly, i-PMT-10 mice displayed lower bacterial loads in the lungs as compared to control mice, after day 63 of intranasal infection and this difference was sustained until the end of the experiment, at 100 days post infection. As for progression of intranasal infection in the spleen, no differences were observed between the 2 groups of mice (Fig. 9b). Histopathological analysis of the lungs, performed at the end point of the experiment (100 days), showed similar areas of inflammatory process in both i-PMT-10 and control mice (Fig. 9c). These data are in line with the ones obtained upon early induction of IL-10 in the intranasal model of infection, although no increase in protection was observed before.

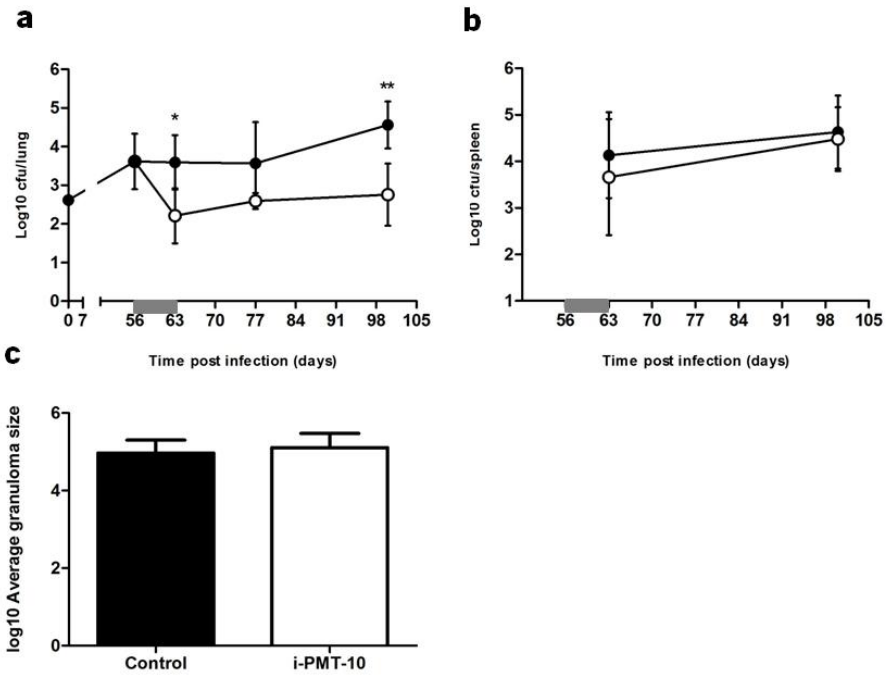

FIGURE 9. Late over-expression of IL-10 increases the resistance of i-PMT-10 mice to intranasal *M. tuberculosis* infection. i-PMT-10 (○) and control littermates (●) were infected intranasally with 2,6 $\log_{10}$ CFU of *M. tuberculosis* H37Rv. i-PMT-10 mice over-expressed IL-10, as shown in figure 8a, during the time-frame indicated (grey bar). Lungs (a) and spleens (b) were removed at the indicated time points, and number of viable bacteria was determined by plating serial dilutions of organ homogenates on Middlebrook 7H11 medium. (c) At 100 days post infection, morphometric analysis of the granulomas was performed in lungs sections of infected mice. Data represent the mean ± SEM from six mice per group. * $p < 0.05$, ** $p < 0.01$.

In contrast, i-PMT-10 mice challenged with *M. tuberculosis* by the intravenous route developed increased bacterial loads at day 72, as compared to control mice, in the liver, spleen and lung (Fig. 10). Interestingly, at day 105 post infection, i-PMT-10 mice were able to decrease the bacterial replication until the levels observed in control mice, therefore suggesting that, in this model, the over-expression of IL-10 induced a transient increase in susceptibility, which was recovered as soon as the effect of IL-10 waned off.

Despite the increased bacterial load in the livers of i-PMT-10 mice, the number (data not shown) and size (Fig. 10d) of granuloma per field at 72 days was similar to that of control mice.

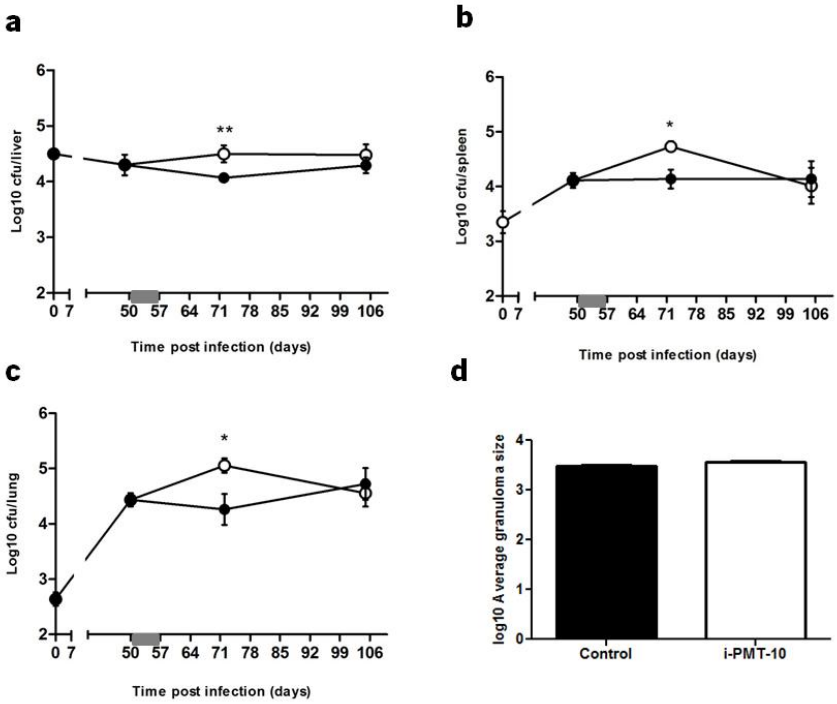

FIGURE 10. i-PMT-10 mice are transiently more susceptible to intravenous *M. tuberculosis* than control littermates. i-PMT-10 (○) and control littermates (●) were infected intravenously with 10^5 CFU of *M. tuberculosis*. i-PMT-10 over-expressed IL-10, as shown in figure 8b, at the time-frame indicated (grey bar). Livers (a), spleens (b) and lungs (c) were removed at the indicated time points, and number of viable bacteria was determined by plating serial dilutions of organ homogenates on Middlebrook 7H11 medium. (d) After 72 days post infection morphometric analysis of average granuloma size was performed in livers sections of infected mice. Data represent the mean ± SEM from five mice per group from one representative of two independent experiments. * $p < 0.05$ and ** $p < 0.01$.

Considering that the over-expression of IL-10 during the late phase of *M. tuberculosis* infection resulted in different outcomes of infection, depending on the route of infection, we will from now on analyze each experiment separately, in an effort to further dissect the observed differences.

A. Intranasal infection

Lungs from intranasally infected i-PMT-10 mice display a lower number of CD4 T cells at 100 days post infection, as compared to control littermates.

To determine whether the increased resistance of i-PMT-10 mice to intranasal *M. tuberculosis* infection was associated with increased cellular responses, we evaluated, by flow cytometry, the dynamics of different cell populations, in the lungs and LN of both induced and control PMT-10 mice.

As shown in figure 11a, after intranasal *M. tuberculosis* infection, the total number of cells in the lungs of i-PMT-10 mice was similar to those of PMT-10, showing that IL-10 is not influencing the overall lung cellularity. However, for several all populations, mainly for CD4 T cells (Fig. 11b), lower numbers were observed throughout the infection for i-PMT-10 mice. These differences reached statistical significance at 100 days post infection, when the number of CD4 T cells was significantly lower in i-PMT-10 mice. Moreover, at this time point, the frequencies of CD4 T cells (Fig. 11c), and, to a higher extent, the frequencies of all myeloid phenotypes (Fig. 11e,g,i), were diminished in the lungs of i-PMT-10 mice. As for the number of cells that accumulated in the LN (Fig. 11j), a slight decrease was apparent at 63 days in i-PMT-10 mice, which was followed by a transient increase at 77 days. This dynamics was not cell type specific, but rather was followed by all the cell phenotypes analyzed (Fig. 11k-m). In addition, no differences were detected in the frequency of cells in the LN between induced and control PMT-10 mice (data not shown).

Taking together, these data demonstrate that the increased protection observed in i-PMT-10 mice to intranasal *M. tuberculosis* infection was not associated with an increased recruitment or expansion of cells in the lungs, or in LN, upon IL-10 induction.

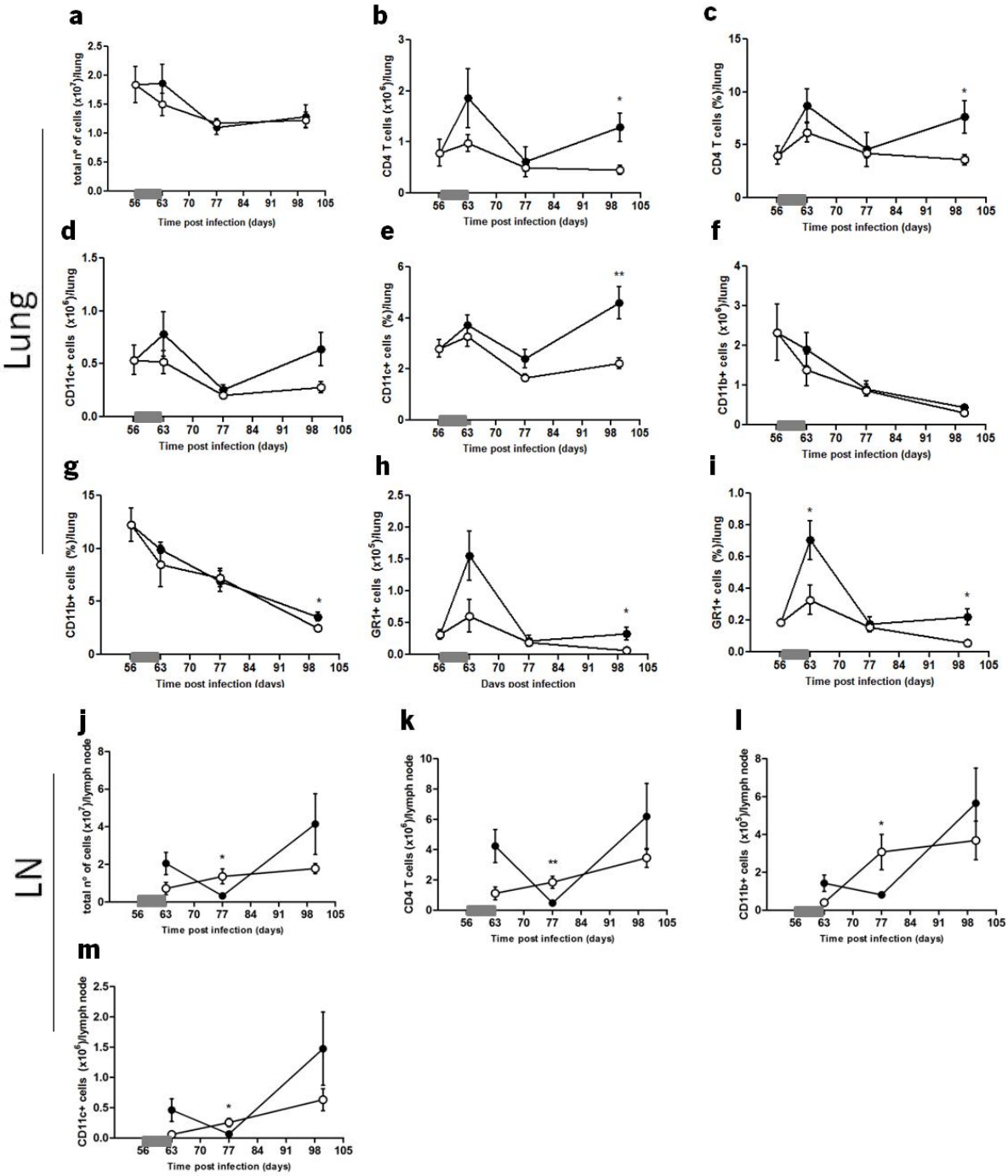

FIGURE 11. Kinetics of cellular expansion in the lungs and LN of intranasally infected mice. i-PMT-10 mice (○) and control littermates (●) were infected intranasally with 2,6 $\log_{10}$ CFU of *M. tuberculosis*. i-PMT-10 over-expressed IL-10, as shown in figure 8a, at the time-frame indicated (grey bar). At different time points, single cell suspensions from lungs (a-i) and LN (j-m) were prepared, stained with antibodies specific for CD4, CD11b, CD11c and GR-1 and analyzed by flow cytometry. The total number of cells of lung (a) and LN (j) was determined using the Newbauer chamber. Data represent the mean ± SEM from six mice per group from one experiment. * $p < 0.05$, ** $p < 0.01$

IL-10 does not affect the expression of IFNγ and TNF after intranasal M. tuberculosis infection.

Although it is extensively described that IL-10 inhibits the IFNγ production by Th1 cells, some studies have reported the ability of IL-10, in combination with other cytokines, like IL-18, to enhance NK cell activity, or, under specific conditions, to stimulate CD4 and CD8 T cells, leading to increased production of IFNγ (147-149). In that sense, we questioned whether the increased resistance of i-PMT-10 mice after intranasal *M. tuberculosis* infection could be related to an additional induction of IFNγ. However, this seemed not to be the case, as no differences were observed in the expression of IFNγ or TNF, in the lungs of i-PMT-10 mice, as compared to control littermates throughout the infection (Fig. 12), suggesting other inflammatory mechanisms responsible for IL-10 mediated increased protection.

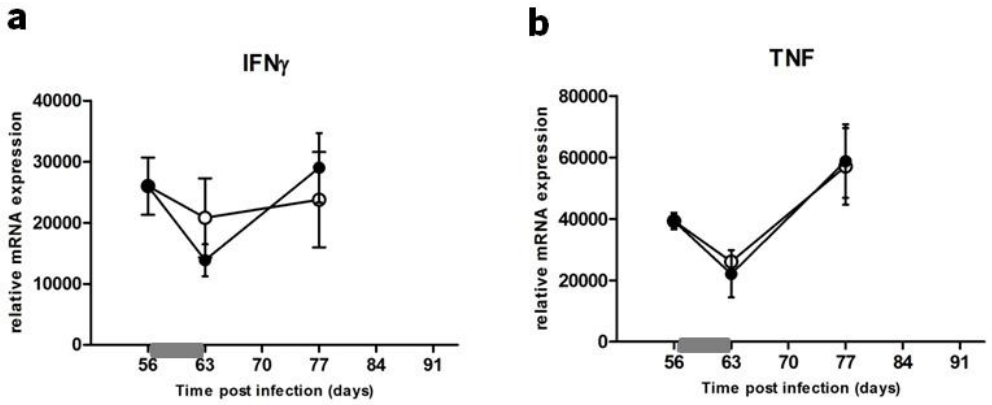

FIGURE 12. Late over-expression of IL-10 does not affect the expression of IFNγ and TNF after intranasal infection. i-PMT-10 mice (○) and control littermates (●) were infected intranasally with 2,6 log10 CFU of *M. tuberculosis*. i-PMT-10 over-expressed IL-10, as shown in figure 8a, at the time-frame indicated (grey bar). Gene expression of IFNγ (a) and TNF (b) was determined in lung homogenates by real time PCR. Data represent the mean ± SEM from six mice per group from one experiment.

LN of i-PMT-10 mice display higher expression of MHC-II than control littermates, late after intranasal M. tuberculosis infection.

The increased capacity of i-PMT-10 mice, to control intranasal *M. tuberculosis* infection was not associated with increased lung pathology, but instead seemed to be associated with a lower number of inflammatory cells with equal ability to produce pro-inflammatory cytokines. One possible explanation for this potentially higher efficacy of i-PMT-10 mice immune response could be a direct effect of IL-10 on the strategies developed by *M. tuberculosis* to subvert the host immunity, and that are believed to contribute to the persistence of infection. For instance, *M. tuberculosis* 19-kDa lipoprotein is one of several PAMPs demonstrated to inhibit MHC-II antigen processing by macrophages after long-term stimulation, affecting the recognition of mycobacteria peptides by effector CD4 T cells (23-25). In addition, it was suggested that mycobacterial inhibition of MHC-II expression in vivo may be more pronounced and effective later in the course of infection (23). In that sense, we investigated whether over-expression of IL-10 affected the expression of MHC-II in the late phase of *M. tuberculosis* infection. To address this, we compared the MHC-II expression in the antigen presenting cells resident in the lungs and LN of both induced and control PMT-10 mice by flow cytometry at 100 days post infection. We gated the antigen-presenting cells based on CD11b and CD11c expression.

Figure 13 shows that CD11b/CD11c cells represent the phenotype with higher MHC-II expression in infected mice, in line with other reports (150). Comparing the 2 groups of mice, it is evident that the expression of MHC-II in all phagocytes was similar in the lungs of both induced and control PMT-10 mice. However, looking at the LN, the MHC-II expression was strongly up-regulated in macrophages and DCs of i-PMT-10 mice, as compared to control littermates. Even though MHC-II up-regulation is not correlative of increased antigen presentation, these data open the possibility that *M. tuberculosis* antigens may be more efficiently presented to T lymphocytes in i-PMT-10 mice during later stages of intranasal infection. Interestingly, this effect of IL-10 might be a consequence of the IL-10 action on immune evasion strategies of *M. tuberculosis*.

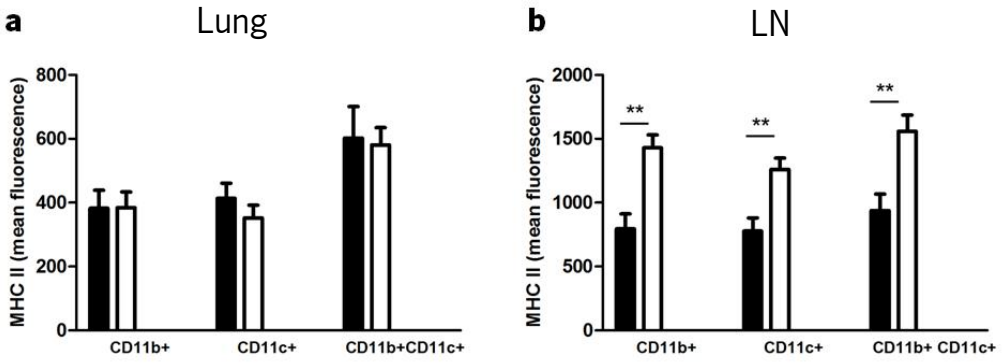

FIGURE 13. After intranasal infection, late over-expression of IL-10 up-regulates surface expression of MHC-II by antigen-presenting cells within the LN. i-PMT-10 mice (white bars) and control littermates (black bars) were infected intranasally with 2,6 $\log_{10}$ CFU of *M. tuberculosis*. After 100 days post infection MHC-II mean fluorescence intensity was analyzed within each of the CD11c/CD11b defined cell subsets from lungs (a) and LN (b) by flow cytometry. Data represent the mean ± SEM from six mice per group from one experiment. ** $p < 0.01$.

Taken together, our results suggest that over-expression of IL-10 induced increased protection of i-PMT-10 mice apparently without increasing the Th1 response. More detailed studies will be needed to determine the mechanisms underlying IL-10 mediated protection.

B. Intravenous infection

i-PMT-10 mice infected intravenously display transient increased number of cells in the spleen

Considering that i-PMT-10 mice infected intravenously with *M. tuberculosis* had increased bacterial loads than control mice at 72 days post infection, we expected to find, at this time point, an impaired cellular response compared to control mice. Flow cytometry analysis of the spleens of infected mice revealed that at 72 days of infection, i-PMT-10 mice had increased number of cells as compared to control littermates, mainly due to an expansion or recruitment of CD4 T cells, GR-1 and CD11c cells (Fig. 14). Because this increased cellularity was found 2 weeks after

the period of IL-10 over-expression by i-PMT-10 mice, the increased infiltration of cells into the spleen is most likely a consequence of the increased bacterial load. In fact, it is possible that an increase in the stimulus (bacterial load) in the spleen signals a potential danger that the immune system tries to overcome. To clarify this issue, further experiments will be required.

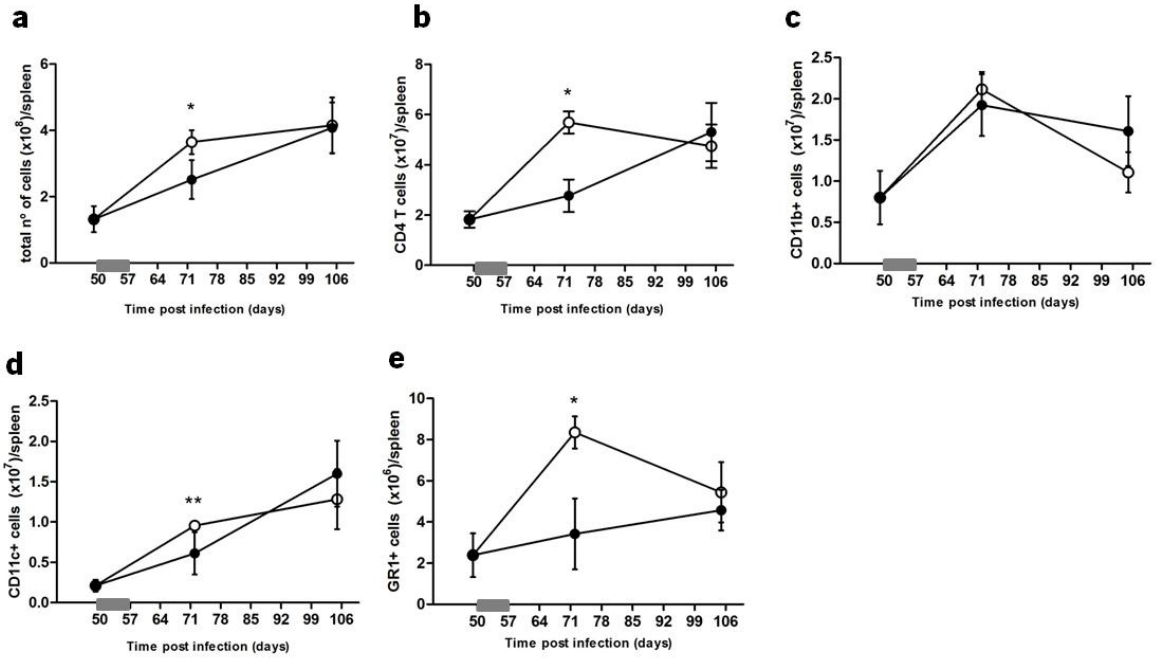

FIGURE 14. Late over-expression of IL-10 induces a transient increase in the expansion of splenocytes. i-PMT-10 mice (○) and control littermates (●) were infected intravenously with 10^5 CFU of *M. tuberculosis*. i-PMT-10 over-expressed IL-10, as shown in figure 8b, at the time-frame indicated (grey bar). At different time points, single cell suspensions from spleens were prepared, stained with antibodies specific for CD4, CD11b, CD11c and GR-1 and analyzed by flow cytometry. Data represent the mean ± SEM from five mice per group from one representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$.

IFNγ and TNF expression is not impaired in the spleens of intravenously infected i-PMT-10 mice

As mentioned before, IFNγ and TNF are key cytokines for the control of *M. tuberculosis* infection. Considering the increase in bacterial load observed when intravenously infected mice were induced to produce IL-10 at later times of infection, we investigated whether this increased growth of *M. tuberculosis* was associated with a decrease in the expression of these key cytokines. Induction of IL-10 expression did not affect the expression of IFNγ or TNF in the spleens of intravenously infected mice at days 72 or 105 post infection, as revealed by real-time PCR (Fig. 15).

These data suggest that the reduced control of intravenous *M. tuberculosis* infection observed in i-PMT-10 mice was not due to an IL-10-mediated abrogation of IFNγ or TNF.

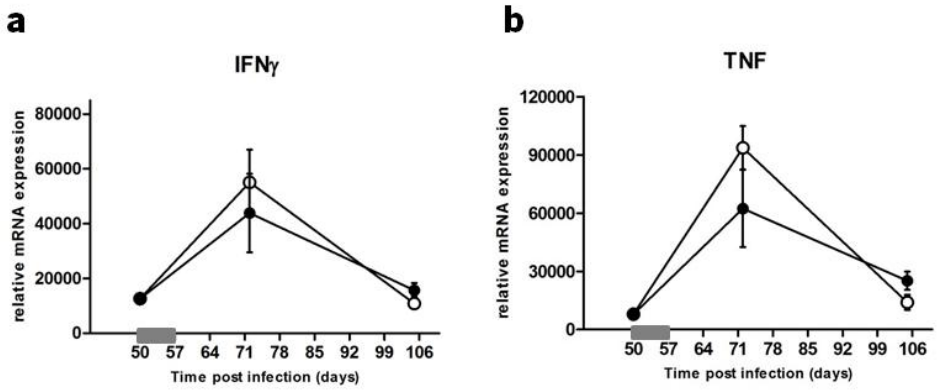

FIGURE 15. Late over-expression of IL-10 does not affect the expression of IFNγ and TNF in the primary local of infection. i-PMT-10 mice (○) and control littermates (●) were infected intravenously with 10^5 CFU of *M. tuberculosis*. i-PMT-10 over-expressed IL-10, as shown in figure 8b, at the time-frame indicated (grey bar). Gene expression of IFNγ (a) and TNF (b) was determined in spleens homogenates by real time PCR. Data represent the mean ± SEM from four mice per group from one representative of two independent experiments.

The expression of iNOS in the granulomas formed upon intravenous infection was not affected by IL-10 over-expression.

The results presented so far show that the increased susceptibility of i-PMT-10 mice to *M. tuberculosis* intravenous infection could neither be attributed to an impaired expansion of leukocytes in the spleen, nor to defective IFNγ and TNF production. Another possibility was that IL-10 could interfere with signal transduction of IFNγ, inhibiting, for example, the expression of iNOS that is observed in macrophage located within granuloma lesions. Indeed, for the efficient control of mycobacteria within macrophages, the induction of iNOS expression is essential (151) as this enzyme catalyses the production of RNI. To test our hypothesis, liver sections from i-PMT-10 and control littermates, at 72 days of infection, were stained for expression of iNOS. Immunofluorescence analysis showed that the frequency of granulomas that were expressing iNOS was similar in both induced and control PMT-10 mice (Fig. 16). Again, IL-10 did not appear to be interfering with macrophage activation in what concerns iNOS expression.

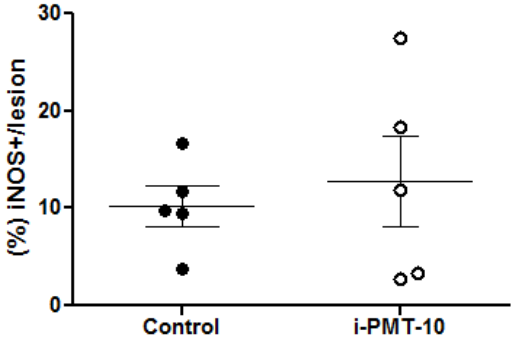

FIGURE 16. Late over-expression of IL-10 did not impair iNOS expression within liver granulomas. i-PMT-10 mice (○) and control littermates (●) were infected intravenously with 10^5 CFU of *M. tuberculosis.* 72 days post infection liver sections were stained for expression of iNOS. Data represent the mean ± SEM from five mice per group from one experiment.

Taken together our results suggest that, after *M. tuberculosis* intravenous infection, an increase on the amounts of IL-10 produced during the late phase of the immune response resulted in a transient increase in mice susceptibility. However, no cellular or cytokine response was found to be impaired by the over-expression of IL-10, but instead we observed, at 72 days post infection, an increased number of cells in the spleen of i-PMT-10 mice, as compared to control littermates. Whether this increased infiltration of cells represents a primary effect of IL-10 or is counterbalancing the enhanced bacterial load we are not certain yet. Nevertheless, at later time points, the immune system was able to reduce the bacterial burden back to the levels reached in the absence of increased IL-10. The precise mechanism that is dyregulated by a sudden increase of IL-10 in the system remains unknown. However, since this mechanism seems to be able to re-establish control of infection, further studies are of importance as this might prove to be a valuable target of immune-modulation during the course of *M. tuberculosis* infections.

4. DISCUSSION

Tuberculosis is one of the most prevalent bacterial infections worldwide and constitutes a leading global health threat (4). Although most people manifest this disease in a latent form, there is an estimated risk of 5–10% of these individuals developing active tuberculosis during their lifetime. Despite the ability of Th1 cell responses to provide protection, full eradication of *M. tuberculosis* is not achieved by the immune response and is even very difficult to accomplish with optimal antibiotic regimens (2, 3). In fact, the predominant mechanisms involved in the reduction of mycobacterial growth, depending on effector CD4 T cells responses, IFNγ, IL-12 and TNF expression are also responsible for tissue damage in the form of granuloma formation and necrosis (77, 144). The balance between host protection and pathogen-induced immunopathologic consequences must, therefore, be maintained for a long period as the lung must resist inflammatory responses to function effectively. However, the pathogen and host-mediated mechanisms that generate and maintain this balanced immune response are poorly understood.

It is noteworthy that *M. tuberculosis* can elicit IL-10 production by monocytes and by T lymphocytes (117, 118, 152, 153). This cytokine was shown, in vitro, to antagonize pro-inflammatory responses against *M. tuberculosis* by down-regulating the production of IFNγ, TNF, and IL-12 (125, 127). Interestingly, although IL-10 has indirectly been implicated in chronicity and progression of tuberculosis, mice deficient for IL-10 show identical growth curves as controls, casting doubt on the validity of the concept that this cytokine would be detrimental for the control of *M. tuberculosis* infection (154). However, a recent study showed that *Il-10* gene disrupted mice succumb suddenly after 6 months of *M. tuberculosis* infection due to an increased Th1 response, suggesting a role for IL-10 in the protection against chronic lung inflammation (132). Other evidences show that IL-10 is not a redundant cytokine regulating host resistance to *M. tuberculosis* infection. Since 1990s, transgenic mice that constitutively over-express IL-10 have been used to investigate the impact of IL-10 in mycobacterial infections (136-140). Although these studies claim that over-expression of IL-10 increases the susceptibility of mice upon infection, the biological mechanism underlying this finding is still not understood. In fact, taking into consideration the pleiotropic properties of IL-10, we believe that murine models that constitutively over-express IL-10 may represent a caveat when it comes to dissect the primary

cause of IL-10 mediated susceptibility. In addition, as mentioned in chapter 1, the early and late periods of the immune response to *M. tuberculosis* have distinct players and dynamics that lead us to speculate that the timing of IL-10 expression could also affect the outcome of infection. In that sense, in the present work, we induced transgenic PMT-10 mice to transiently over-express IL-10 either during the onset of the innate immune response to *M. tuberculosis*, or during the established acquired immunity, in which the bacterial load is already controlled and maintained. The impact of IL-10 was evaluated after intranasal or intravenous models of infection.

Our results demonstrate that high amounts of IL-10 produced early after *M. tuberculosis* infection do not impact the outcome of infection. Indeed, i-PMT-10 mice, induced to over-express IL-10 early after intranasal or intravenous challenge with *M. tuberculosis,* had the same capacity as non-induced littermates to control the infection (Fig. 2). We also observed that IL-10 failed to impair the production of TNF and IFNγ (Fig. 6), which are key cytokines for the resolution of *M. tuberculosis* infection. This fact may be the major reason for the ability of i-PMT-10 mice to control the infection as the majority of the antimicrobial mechanisms induced in macrophages to kill mycobacteria are triggered by IFNγ.

Why was IL-10 not able to impair the production of pro-inflammatory cytokines? One possible explanation is that IFNγ is poorly induced until 14 days of infection, and therefore, the very early expression of IL-10 was not long enough to suppress the kinetics of IFNγ and TNF production in the course of infection. In fact, it has been suggested that after aerosol or intranasal exposure, the induction of the host effector mechanisms to inhibit *M. tuberculosis* growth is relatively slow, when compared with responses to other pulmonary pathogens (155-157). Whether this slowness reflects an immunomodulatory activity of *M. tuberculosis* is not yet defined. In line with this, *Mogues et al* showed that important effector molecules for *M. tuberculosis* clearance fail to impair bacterial growth until the third week of infection, when IFNγ-producing T-cells arrive to the primary site of infection (158). In that study, the authors compared the course of *M. tuberculosis* infection in the lungs of wild-type mice and gene deleted mice incapable of producing iNOS, MHC class II or IFNγ. Interestingly, until the third week of infection, the growth rate of *M. tuberculosis* was similar in all mice groups.

Therefore, it is conceivable to speculate that the effector innate immunity against *M. tuberculosis* is not fully triggered until T cell responses are elicited, and thus, an early putative modulation by IL-10 is not sufficient to impact the outcome of infection.

Migration of DCs to draining LN, a process that seems to be mediated by IL-12p40, is required for the activation of naïve T cells (159). We anticipated that high levels of IL-10 would inhibit or delay the priming and generation of T cell responses, in a way that would affected the bacterial control during the subsequent process of acquired immunity. Since it has been shown that *M. tuberculosis* disseminates from the lungs to the LN around day 10 post infection (145), we induced PMT-10 mice to over-express IL-10 between days 4 to 13 after intranasal *M. tuberculosis* infection. We showed that over-expressing IL-10 during this critical period of *M. tuberculosis* infection did not impair significantly the activation of CD4 T cells in the LN (Fig. 6b), even though we detected a slight delay in the expansion of leukocytes at 14 days of infection (Fig. 4f). In addition, over-expression of IL-10 did not hamper the expansion of cells in the lungs, but instead induced a later increased inflammatory response at 28 days post infection, characterized by increased frequency of activated CD4 T cells (Fig. 4b) and enhanced TNF expression (Fig 7b).

Whether this later increase of inflammatory response was irrelevant or fundamental for the control of *M. tuberculosis* remains to be elucidated. In that sense, it is possible that i-PMT10 mice displayed similar pulmonary bacterial loads as control littermates, due to timely readjustment of the immune system, perhaps in parallel with a slow growing bacillus (160).

In a previous study, *Demangel et al*, showed that BCG infected IL-10$^{-/-}$ DCs migrated to the draining LN more efficiently than wild-type DCs, suggesting that autocrine IL-10 is an important regulator of DCs migration in vivo in response to microbial stimuli (161). However, in that study, it was not shown whether this early arrival of DCs into the LN resulted in an early protective immune response in the lungs, improving the outcome of infection.

Nevertheless, we can conclude that during the early immunological events of *M. tuberculosis* infection, and independently of the route of inoculation, high amounts of IL-10 do not affect the ability of the host to control the infection.

In the second part of the work we evaluated the impact of high levels of IL-10 during the chronic phase of *M. tuberculosis* infection.

We showed that after *M. tuberculosis* intravenous infection, increased amounts of IL-10 during the late immune response resulted in a transient increase of mice susceptibility to *M. tuberculosis* (Fig. 10). Although *M. tuberculosis* persistence in chronically infected mice is characterized by an equilibrium, in which the rate of bacterial cell division is slow (162, 163), our results showed that a short period of immunossupression induced by IL-10, is rapidly taken in advantage by the pathogen for increased bacterial replication. In other studies, re-growth of *M.*

tuberculosis after stationary level of infection occurred by delivering to mice inhibitors of Th1 immunity, as anti-CD4 antibody (164), anti-TNF antibodies (165), or inhibitors of iNOS (166). However, in our work, we could not detect any impairment of cellular (Fig. 14) or cytokine (Fig. 15) responses induced by IL-10 over-expression, nor decreased iNOS expression in the granulomatous tissue (Fig 16). On the contrary, we observed, at 72 days, increased number of cells in the spleen of i-PMT10 mice (Fig 14), compared to control littermates. It is possible that we missed an earlier down-regulation of the immune response caused by IL-10, as the increased infiltration of cells into the spleen resembles our previous data, in which after IL-10 boost, a delayed expansion of cells was frequently detected in the lymphoid organs. In addition, the fact that later on, at 105 days post infection, i-PMT10 mice were again able to control the bacterial loads, reinforces the hypothesis that, at 72 days, the immune system was already mounting a stronger inflammatory response. However, we cannot exclude other scenarios that may have occurred at 72 days, as IL-10 can impair other pathways important for bacterial control, such as apoptosis or in situ production of RNI (79). These hypothesis will be the focus of further studies.

In previous studies (136, 140), a continuous over-expression of IL-10 led to increased susceptibility to *M. tuberculosis* that was maintained throughout the infection. Here we showed that after an IL-10 boost, the host immune system alone is skilled to mount a stronger and organized response to balance the exceeding bacterial replication. Interestingly, i-PMT10 mice regained the control of bacterial growth until the same bacterial load observed before the IL-10 challenge. This fact suggests that regulatory mechanisms followed in time the increased pro-inflammatory response, as a mean to maintain lung function, implying that this CFU level represents the best compromise between tissue damage and bacterial persistence.

This plasticity of the immunocompetent host can be advantageous in the setting of human diseases. IL-10 has been considered a promising candidate for therapeutic use based on its immunomodulating activities in animal models of acute and chronic inflammation, autoimmunity and cancer (79). Thus, several large clinical trials are being performed, testing multiple IL-10 dosages in patients (95). However, an outstanding concern is the possibility of IL-10 therapy to induce the recrudescence of latent tubercle bacilli in asymptomatic patients. In the past, the introduction of TNF blockers (etanercept, infliximab and adalimumab) have contributed greatly to the control of rheumatoid arthritis, however the treatment was also associated with increased reactivation of latent tuberculosis (167, 168). Regarding IL-10 modulation, we showed that a transient increase on IL-10 expression can lead to increased susceptibility to *M. tuberculosis*,

which can be reverted by bringing IL-10 back to basal levels. In that sense, IL-10 therapy in patients with both inflammatory disease and latent tuberculosis could be accomplished, yet with careful surveillance. In line with this, as future work, we are planning to evaluate the capacity of i-PMT-10 mice to recover the bacterial control after repeated IL-10 boosts during the chronic phase of *M. tuberculosis* infection.

The infection model that is presented in this work, where mice of a relatively resistant strain (C57BL/6) are infected via intranasal or intravenous route, share some important characteristics with human infections, by the evidences that chronic infection is controlled by the immune response, granulomas are present in the lungs and the mice do not show overt signs of disease (169). However, it is accepted that the bacterial load that persists in the lungs of infected mice is higher than the one observed in latent disease in humans. It is not known, however, to what extent this fact limits the experimental murine studies of tuberculosis. Still, this difference was partially resolved, with the development of the Cornel Model, which is a murine model of latent tuberculosis that consists in using chemotherapy to reduce the bacterial burden once the infection is established. After cessation of the drug treatment, the infection is induced to reactivate by immunossupression with steroids (164). Taking this in consideration, we are interested, in future work to evaluate the role of IL-10 in reactivation of latent tuberculosis by performing the Cornell model in PMT-10 mice. In that sense, in parallel with the use of steroids, immunossupression would also be induced by over-expression of IL-10 in chronically infected PMT-10 mice.

In contrast with the intravenous infection model, over-expression of IL-10 during the late phase of intranasal infection increased the resistance of i-PMT-10 mice until the end of the experimental period (Fig 9a). This result was surprising, considering that the role of IL-10 in infectious diseases seems to be associated to a down-modulation of inflammatory responses to microbial pathogens. However, the apparent paradoxical nature of IL-10 had previously been described in other experimental models.

A large number of studies have addressed the involvement of IL-10 in tumor immunity and contradictory results have been reported. Studies employing tumor cells transfected with IL-10 or IL-10 transgenic mice demonstrated increased tumor growth in vivo, while others showed decreased growth potential (149, 170-180). These studies suggested that lower levels of IL-10 may result in immunossupression and enhanced tumor growth, while high levels of this cytokine

may result in immune activation and tumor rejection. In fact, some IL-10 activities specifically contribute to inhibition of tumor growth, by stimulating cytotoxic cells function (181-183), by increasing the sensitivity of target cells to NK-mediated lysis (176, 184), and enhancing antibody-mediated immunity (175). On the other hand, IL-10 can have immunosuppressive effects on anti-tumor immunity, or behave as a growth factor for certain tumor cells (6, 185, 186).

Moreover, studies evaluating the efficacy of IL-10 to suppress the expression and secretion of IFNγ in patients with Crohn's disease showed both stimulatory and inhibitory effects of IL-10 depending on the dose of administration. *Tilg et* al (148) showed that a high dose of IL-10 (20μg/kg) administrated subcutaneously, surprisingly, induced elevation of IFNγ and the inflammatory marker neopterin in the blood of patients with Crohn's disease, on the other hand, *Van Deventer* et al. (187), showed that the most favorable clinical responses were observed with lower doses of IL-10 (5μg/kg).

Finally, a small number of studies reported the capacity of IL-10 to stimulate the immune response against pathogens. *Kurilla et al* (188) showed that IL-10 enhances NK cell mediated resistance against vaccinia virus infection in vivo. Furthermore, *Neyer et al* reported that mice lacking the gene for IL-10 displayed less NK cytotoxic activity when compared with wild-type mice following infection with *T. gondii* (189). *Cai et al.* (147) suggested an indirect mechanism by which IL-10 stimulates NK activity, claiming that IL-10 enhances the ability of IL-18 to stimulate NK cell production of IFNγ.

What determines which of the IL-10 activities will dominate in an immune response is not yet defined. Nevertheless, it is recognized that the ultimate consequences of IL-10 expression or therapy depend on many variables, such as IL-10 levels, source, timing and duration of expression.

It is conceivable, therefore, that high levels of IL-10 during one week of chronic tuberculosis infection induced additional immunological pathways that culminated in increased protection. Comparing to the intravenous infection, other variables might be determinant for the distinct outcomes, such as the local of infection, the dynamic of cellular response that characterize each infection, and the levels of IL-10 that were produced. Over-expression of IL-10 induced similar kinetics of expansion of cells in the lymphoid organs of intranasal and intravenous infection. However, the fact that the lung is the primary local of infection in intranasal infection may account for the different outcomes. In addition, we did not compare the levels of IL-

10 in the different organs, nevertheless, the serum levels of IL-10 were increased in mice challenged by the intranasal route as compared to the intravenous route. These variations may therefore be responsible for the different outcomes of infection observed.

Analyzing the results of the intranasal experiment, it is evident that the stimulatory effect of IL-10 in i-PMT-10 mice was not correlated with increased production of IFNγ, nor TNF (Fig 12) in the primary local of infection. Although the resistance to *M. tuberculosis* is highly dependent on the ability of the host to produce IFNγ, apparently an increased Th1 response was not causative of decreased bacterial loads in i-PMT-10 mice. This evidence is in accordance with the work of *Fonseca et al* (190), which evaluated the capacity of vaccine adjuvant CpG, which induces strong Th1 biased responses, to confer protection against *M. tuberculosis* infection. These authors concluded that immunization of mice with CFP (culture filtrate protein) antigens, combined with CpG, conferred less protection than immunization with CFP combined with incomplete Freund's adjuvant, as a result of severe pulmonary injury and necrosis. Moreover, as already pointed out, IL-10 $^{-/-}$ mice generate an excessive Th1 response after *M. tuberculosis* infection, but, die earlier than wild-type mice (132).

The increased resistance of i-PMT-10 mice was also not related to increased infiltration of cells to the lung (Fig. 11a-i), as the number of CD4 T cells, macrophages and DCs, throughout the infection, was similar or diminished when compared to control littermates. Only a transient increase of cells at 77 days post infection was observed in the LN of i-PMT-10 mice (Fig. 11j-m). However, before that time, lungs of i-PMT-10 mice had already decreased bacterial loads compared to control mice. Moreover, morphometric analysis of the granulomatous (Fig. 9c) response in the lungs of infected mice suggested that i-PMT10 mice did not develop increased pathology than control littermates. This is in contrast with other studies, in which decreased bacterial burdens after immune modulation was associated with pathologic consequences that affected the long-term survival of the mice (191).

This better control in apparent absence of immunopathology in the lungs of i-PMT-10 mice highlights the efficiency of the macrophages to kill *M. tuberculosis*. Certainly, the next question is to know what are the mechanisms underlying this increased protection. However, this is not a "straightforward approach", as the factors that prevent the immune system to eradicate *M. tuberculosis* during chronic infection are not yet defined, and there are no useful markers of protection to this disease. Nevertheless, it has been suggested that *M. tuberculosis* develops

strategies to avoid or subvert the effector mechanisms of the macrophage (3, 169, 192). One of the mechanisms proposed to contribute to the persistence of the tubercle bacillus in the host is the attenuation of the process of antigen processing and presentation by macrophages and DCs (150, 193). Interestingly, *Noss et al* (23) reported that the inhibition of the MHC-II antigen presentation pathway by *M. tuberculosis* 19-kDa lipoprotein occurs at later time points after exposure to the lipoprotein. Based on these results, the authors suggested that this interaction allows *M. tuberculosis,* in a chronic tuberculosis lesion, to evade detection by CD4 T cells, enabling the establishment of a persistent infection. Interestingly, we found that macrophages and DCs residing in the LN of i-PMT-10 mice in the late stage of *M. tuberculosis* infection displayed increased MHC-II expression, as compared to control mice. Nevertheless, this result alone, although suggestive, is not indicative of better antigen processing and presentation of *M. tuberculosis* antigens. In addition, it was not possible to distinguish *in vivo* if the antigen presenting cells with increased MHC-II expression were the ones that harbored mycobacteria, a limitation that can be overcome by infecting PMT-10 mice with GFP-expressing *M. tuberculosis*.

Nevertheless, other macrophage antimicrobacterial mechanisms seem to be modulated by *M. tuberculosis*, and once improved by the host could decrease chronic bacterial persistence, such as the generation of RNI (40, 41), the delivery of bacilli-containing phagosome to the lysosomal compartment (14), the manipulation of host cell metabolism (e.g. cholesterol, iron) and even the spatial organization of the granulomatous structures, such as the physical separation of infected macrophages in the granuloma center from the peripheral lymphocytes (194). To our knowledge, none of these mechanisms have been described to be potentiated by IL-10 production.

As mentioned above, besides increased IFNγ production, high amounts of IL-10 have been associated with enhanced cytotoxic activity of NK and CD8 T cells responses. Whereas the role of NK cells seems to be confined to the early immune response to *M. tuberculosis* infection, *Einarsdottir et al* showed recently that cytotoxic CD8 T cells specific for *M. tuberculosis* peak in the lungs of infected mice at about 4 weeks of infection and decrease slightly with time (65). In our model, could IL-10 elicit enhanced CD8 cytotoxic activity later in *M. tuberculosis* infection? Even though in i-PMT-10 mice the number and frequency of CD8 T cells were not increased in the course of infection (data not shown), we did not measure the cytotoxic activity of these cells,

which includes induction of apoptosis via the Fas-FasL pathway and killing via perforin and granulysin (195).

Importantly, recent in vitro studies from our laboratory showed that high concentrations of IL-10 in the presence of IL-17 result in an increased control of mycobacterial infection (*A. Cruz et al, unpublished*). In this work, bone marrow derived macrophages from IL-10$^{-/-}$ mice were infected with BCG and treated with different doses of IL-10 combined with an invariable dose of IL-17. Macrophages treated with IL-17 and very high concentrations of IL-10 displayed decreased bacterial loads when compared to non-treated macrophages. Moreover, this effect was not observed when macrophages were treated with very high concentrations of IL-10 alone. These results suggest that the ratio IL-17/IL-10 strongly affects the capacity of macrophages to control mycobacterial infections and opens the possibility that the increased protection of i-PMT-10 mice was correlated with the interplay between IL-17 and high amounts of IL-10. It would be interesting to test this hypothesis in future work.

5. CONCLUSION

The present work demonstrates for the first time that the role of IL-10 in *M. tuberculosis* infection must be evaluated in the context of the amount and timing of IL-10 production, as well as the route of inoculation, otherwise, misleading or contradictory results will certainly arise. The different outcomes in *M. tuberculosis* infection observed in this work highlight the unrevealed pathogenesis of *M. tuberculosis in vivo* infection, demonstrating that further fundamental research is needed before designing more effective vaccines or therapeutic strategies. Therefore, understanding how IL-10 interplays with the complex relationship between the host and mycobacterial components that modulate the outcome of *M. tuberculosis* infection will be a challenge for more detailed studies.

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